Structural Basis of Ion Pumping by Ca^{2+}-ATPase

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Ca^{2+}-ATPase, first identified in the 'relaxing factor' of muscle contraction by Ebashi and Hasselbach, gave rise to the calcium theory that Ca^{2+} is a fundamental and ubiquitous factor in the regulation of intracellular processes. In the past 25 years or so I have been struggling with this ATPase (1). Since the success of the first crystallisation of SERCA1a in 2000 (2), more than 10 reaction intermediates that roughly cover the entire reaction cycle have been crystallised and allowed us to understand the mechanism of ion pumping. Using recombinant proteins produced with an adenovirus-COS expression system, we can now address the structures of other SERCA isoforms and the regulatory mechanism by phospholamban/sarcolipin (3). Furthermore, by developing a technology for visualising lipid bilayers, we now begin to understand how Ca^{2+}-ATPase interacts with phospholipids as an integral component of the pumping mechanism (4). In this lecture, I would like to overview our current understanding of the mechanism of SERCA pumps through their atomic structures.

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Signaling to Transcription: the Calcium-Calcineurin-NFAT Pathway in T Cells

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The transcription factor NFAT (nuclear factor of activated T cells) is a major target of calcium signaling in T cells. NFAT is normally cytoplasmic and heavily phosphorylated in resting cells; calcium influx through ORAI-STIM store-operated calcium channels leads to its dephosphorylation by the calcium/calmodulin-dependent phosphatase calcineurin. Once in the nucleus, NFAT directs both the transcriptional program of T cell activation and an opposing process in which it turns on a negative feedback program that attenuates immune function. The hyporesponsive state induced by this negative feedback program has been termed T cell "anergy" or "exhaustion", and is essential to ensure that activated T cells do not go on to trigger an autoimmune attack on the host's own tissues. However, the negative feedback program that induces the hyporesponsive state is counterproductive in cases when prolonged T cell function is needed.

Among the many negative regulators induced in hyporesponsive T cells are inhibitory cell-surface receptors such as PD-1, LAG-3, CTLA-4, and TIM-3. Blocking antibodies against these inhibitory receptors mitigate hyporesponsiveness and promote tumor rejection, and checkpoint blockade therapies that involve treatment of patients with cancer with blocking antibodies to these inhibitory receptors, as well as CAR-T cell therapy in which a patient's own T cells are mobilized against tumor antigens, have begun to show remarkable promise in the clinic.

Recent advances in whole-genome sequencing technologies have allowed scientists to probe the changes in chromatin structure and the accompanying changes in gene transcription that occur during biological immune responses. Using mouse models of viral infection and cancer, we have used several such systems to study the role of NFAT in immune responses molecular detail: the differentiation of naïve CD8+ T cells into effector and memory cytolytic T cells (CTLs) during acute viral infections, the development of CD8+ T cell exhaustion during chronic viral infection; the process by which CD8+ T cells become first activated and then exhausted when they infiltrate tumors.

Ryanodine Receptor Structure and Function

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Ryanodine receptor (RyR) channels are required for release of calcium from intracellular stores, a process essential for many cellular functions including excitation-contraction (EC) coupling in skeletal and cardiac muscle, and hormone and neurotransmitter release. They are the largest ion channels, comprised of the four identical ~565 kDa channel-forming protomers, as well as regulatory subunits, enzymes and their respective targeting/anchoring proteins, in a macromolecular complex that exceeds three million daltons. We have obtained high-resolution cryo-electron microscopy (Cryo-EM) reconstructions from highly purified rabbit skeletal muscle RyR1 in the open and closed states. Our data reveal that RyRs are members of the six transmembrane family of ion channels and show a mechanism for channel gating suggesting that calcium binding facilitates mechanical coupling of conformational changes in the cytosolic region to opening of the channel gate. In heart failure and myopathies, the RyR channel is excessively phosphorylated, oxidized and nitrosylated and depleted of the RyR-stabilizing protein calstabin (FK506 binding protein 12/12.6). This remodeling of the RyR channel complex results in an intracellular SR Ca^{2+} leak and impaired contractility and defective learning and memory. A novel class of RyR-stabilizing drugs, RycalsTM, which reduce Ca^{2+} leak by stabilizing the RyR channels due to preservation of the RyR-calstabin interaction, have recently been shown to improve contractile function in both heart and skeletal muscle and to have anti-arrhythmic activity in animal models of atrial fibrillation. This opens up a novel therapeutic strategy for the treatment of contractile failure in disorders of cardiac and skeletal muscles. Conflict of interest: A.R. Marks is a consultant for a start-up company, ARMGO Pharma Inc., that is targeting RyR channels to treat heart disease and to improve exercise capacity in muscle diseases.

Regulation of Calcium Release Through Inositol 1,4,5-trisphosphate Receptors

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The modulation of intracellular calcium concentration [Ca^{2+}]i is a signal used by all living organisms to control a variety of cellular processes, including gene transcription, regulated secretion, proliferation, muscle contraction, fertilization, and apoptosis. Inositol 1,4,5-trisphosphate (IP_3) receptors (IP_3Rs) are ligand-gated calcium channels present in most eukaryotic species and are primarily responsible for Ca^{2+} signals that control these processes. There are three homologous isoforms of IP_3R (denoted as IP_3R1, IP_3R2, and IP_3R3), often with overlapping distribution in mammalian tissues. Mutations in these proteins are increasingly been associated with human disease including spinocerebellar ataxia, Gillespie syndrome and generalized anhydrosis. The individual channel is assembled with a tetrameric architecture consisting of homo- or heteromeric subunits and is localized in the membranes of intracellular calcium stores. Ca^{2+} release through IP_3R can be regulated by many cellular events including phosphorylation, adenine nucleotides, protein binding partners and proteolytic cleavage. These modulatory events are important in encoding the distinct spatiotemporal characteristics of the signal necessary for the precise activation of downstream effectors. Research from the lab studying IP_3R regulation and its functional consequences both in native receptors and mutants associated with human disease will be presented.

Sticking to Acidic Ca^{2+} Stores

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Mobilisation of stored calcium is a ubiquitous mechanism for regulating cell function. The prevailing view is that these calcium stores reside in the endoplasmic reticulum. But our work has advanced the concept that the endo-lysosomal system and other acidic organelles are potentially equally important, non-canonical stores of calcium that physically associate with the endoplasmic reticulum. Central to signalling through these so called 'acidic calcium stores' is the calcium mobilizing messenger, NAADP and its endo-lysosomal target, the two-pore channels (TPCs). Here, I discuss the emerging involvement of membrane contact sites, regions of close apposition between organelles that facilitate information transfer. I focus on a new mechanism whereby NAADP-dependent calcium release through TPC1 maintains contact between endosomes and the endoplasmic reticulum to temper signalling by an internalised growth factor. Endosome-endoplasmic reticulum contacts thus emerge as calcium-dependent hubs for signalling.

The Role of Acidocalcisomes in Phosphate Homeostasis and Ca^{2+} Signaling

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Acidocalcisomes are acidic calcium stores rich in polyphosphate. Polyphosphate is a polymer of three to hundreds of orthophosphate monomers linked by high-energy phosphoanhydride bonds similar to those present in ATP. Acidocalcisomes have been conserved from bacteria to human cells and were originally named after their discovery in trypanosomatids. The acidic pH of acidocalcisomes is maintained by a vacuolar H+ pyrophosphatase, a vacuolar H+-ATPase, or both, in bacteria and protists, while in other eukaryotic cells a V-H+-ATPase is required. Acidocalcisomes of several protists possess a Ca^{2+}-ATPase for Ca^{2+} uptake. Ca^{2+} release from acidocalcisomes of trypanosomatids is through a Ca^{2+} channel, the inositol 1,4,5-trisphosphate receptor. Because polyphosphate is a polyanion its charges are balanced by several organic (basic amino acids, polyamines) and inorganic (sodium, potassium, calcium, zinc, iron) cations and acidocalcisomes possess transporters for these cations that have been identified in recent proteomic studies. Eukaryotic acidocalcisomes belong to the group of lysosome-related organelles. They have a variety of functions, from the storage of cations and phosphorus to calcium signaling, autophagy, osmoregulation, blood coagulation, and inflammation. Two organelles in mammals, the platelet dense granules and the mast cell granules, have characteristics in common to acidocalcisomes of protists. The biogenesis of these organelles in both protists and mammalian cells involves the function of the adaptor protein 3 (AP-3) complex, as is typical of lysosome-related organelles. Synthesis and translocation of polyphosphate in acidocalcisomes of trypanosomatids and in the acidocalcisome-like vacuole of yeast is by the vacuolar transporter chaperone complex (VTC), while phosphate release is through a phosphate/Na+ symporter (Pho91) that is stimulated by inositol polyphosphates. In trypanosomatids acidocalcisomes interact with the mitochondria and the contractile vacuole complex.

Effects of CICR Activity and Expression Levels of RyR2 on Ca^{2+} Homeostasis in HEK293 Cells

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Background: Type 2 ryanodine receptor (RyR2) is the Ca^{2+} release channel on sarcoplasmic reticulum gated by Ca^{2+}-induced Ca^{2+} release (CICR) mechanism. Mutations in RyR2 have been implicated in various arrhythmogenic disorders such as catecholaminergic polymorphic ventricular tachycardia and idiopathic ventricular fibrillation. To date, gain-of-function (GOF) mutants with increased CICR activity and loss-of-function (LOF) mutations with decreased CICR activity have been reported. In addition, changes in expression level of RyR2 have been also correlated to cardiac diseases. Because the density of RyR2 would also affect net Ca^{2+} flux rate through ER, it is important to know the effects of changes in expression level and CICR activity of mutant RyR2 on cellular Ca^{2+} homeostasis. In this study, we examined how expression level and CICR activity of RyR2 affect Ca^{2+} homeostasis using HEK293 cell expression system.

Methods: Expression of WT and mutant RyR2s were induced by doxycycline. Protein levels were determined by Western blot analyses. CICR activity of WT and mutant RyR2s was determined by [3H]ryanodine binding assay. Cytoplasmic Ca^{2+} ($[Ca^{2+}]$cyt) and ER Ca^{2+} ($[Ca^{2+}]$ER) were monitored with G-GECO1.1 and R-CEPIA1er, respectively.

Results and discussion: WT and mutant RyR2 protein similarly increased with time after induction and reached steady state at ~24 h. After 24hr of induction, WT and GOF mutant cells showed spontaneous periodic increase in $[Ca^{2+}]_{cyt}$ (Ca^{2+} oscillation) and corresponding periodic decrease in $[Ca^{2+}]_{ER}$. The upper level of the $[Ca^{2+}]_{ER}$ (threshold $[Ca^{2+}]_{ER}$) was lower in GOF mutants than in WT. There was a good inverse-correlation between threshold $[Ca^{2+}]_{ER}$ and CICR activity. At shorter periods after induction, threshold $[Ca^{2+}]_{ER}$ was higher than that at 24 hours in both WT and GOF. There was a good inverse-correlation between threshold $[Ca^{2+}]_{ER}$ and expression level. Our results indicate that $[Ca^{2+}]$ER is determined by both CICR activity and expression level of RyR2.

Evolution of Ca^{2+} Signalling

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Is evolution a theory, a system, or a hypothesis? It is much more – it is a general postulate to which all theories, all hypotheses, all systems must henceforward bow and which they must satisfy in order to be thinkable and true".

Pierre Teilhard de Chardin,

All living cells maintain exceptionally low concentration of free Ca^{2+} ions in their cytosol; this is a universal attribute of life in the Earth. Extremely steep trans-plasmalemmal gradient for Ca^{2+} sets the background for utilisation of Ca^{2+} ions as iniquitous and pluripotent signalling molecules that regulate numerous cellular processes. To create and maintain low cytosolic Ca^{2+} concentration numerous transporting molecules are required and it is hard to conceive that the very first cells were in possession of these molecules from their very emergence which happened ~3.5 billion years ago.

Eukaryotes have inherited pumps and antiporters and expanded their deployment from plasma membrane to intracellular organelles; similarly Ca^{2+}-binding proteins become available to some of these intracellular compartments. This allowed highly localised control over Ca^{2+} in cells of continuously increasing size and complexity. This is particularly true of compartments involved in trafficking (for example the endoplasmic reticulum) function of which is largely governed by calcium. Probably evolution of complex cell structure was going in parallel with the evolution of Ca^{2+} signalling. In eukaryotes, Ca^{2+} has, thus, become a dominant regulator of intracellular vesicle traffic. This had to be "invented", not only for influx under widely different regulation principles – modification by extracellular and intracellular signals – but also for mobilization of Ca^{2+} from intracellular stores and vesicles undergoing trafficking.

At the end of this long lasting evolutionary journey the sophisticated and coordinated Ca^{2+} signalling system became omnipresent. Besides Ca^{2+} pumps and transporters this system includes Ca^{2+} channels responsible for fast and topologically defined Ca^{2+} diffusion across plasma membrane and endomembranes.

S100A4 Regulates Macrophage Invasion by Distinct Myosin -Dependent and Independent Mechanisms

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In experimental models of breast cancer, stromal macrophages regulate multiple steps in the metastatic cascade. Specifically, tumor-associated macrophages enhance breast tumor cell invasion and intravasation, and metastasis-associated macrophages enhance the seeding and colonization of breast tumor cells at the lung. S100A4, a member of the S100 family of Ca^{2+}-binding proteins, is a key regulator of cell migration and invasion. Although S100A4 expression in tumor cells clearly induces a metastatic phenotype, S100A4 expression in stromal cells within the tumor microenvironment has an equally important role in promoting tumor progression. Our previous studies showed that bone marrow-derived macrophages from $S100A4^{-/-}$ mice exhibit defects in directional motility and chemotaxis in vitro, as well as reduced recruitment to sites of inflammation in vivo (Li et al., Mol Biol Cell 21:2598, 2010). We now show that the loss of S100A4 produces two mechanistically distinct phenotypes with regard to macrophage invasion: a defect in matrix degradation, due to a disruption of podosome rosettes caused by myosin-IIA overassembly, and a myosin-independent increase in microtubule acetylation, which increases podosome rosette stability and is sufficient to inhibit macrophage invasion. Our studies point to S100A4 as a critical regulator of macrophage-mediated matrix degradation, whose actions converge on the dynamics and degradative functions of podosome rosettes.

The IP_3 Receptor and the Ryanodine Receptor in the Regulation of Autophagy

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The Ca^{2+} ions released from the endoplasmic reticulum (ER) by the inositol 1,4,5-trisphosphate (IP_3) receptors (IP_3Rs) and the ryanodine receptors (RyRs) control a plethora of cellular functions. Recently it was recognized that these intracellular Ca^{2+}-release channels are also involved in the regulation of autophagy. The latter is a conserved catabolic process, which has a prosurvival function and which is upregulated in stress situations. Basal ER-tomitochondria Ca^{2+} flux mediated by the IP_3R is essential for mitochondrial bioenergetics and limits autophagy. In contrast herewith, starvation-induced or rapamycin-induced autophagy are dependent on IP_3-induced Ca^{2+} release. We now investigated whether autophagy induced by other mechanisms would also be dependent on Ca^{2+} signaling and/or on the IP_3R and whether the RyR also is involved in autophagy control.

Autophagy induced by the proline analog L-azetidine-2-carboxylic acid (AZC) or by the polyphenolic phytochemical resveratrol appeared inhibited by BAPTA-AM. Interestingly, resveratrol-induced autophagy was abolished in HEK 3KO cells lacking all three IP_3R isoforms (provided by D. Yule, Univ. Rochester, USA) but in HEK 3KO cells stably re-expressing IP_3R1, the autophagic response was fully recovered. Moreover, the altered autophagic response could not be attributed to a modulation of the mTOR/p70S6K pathway. Although acute addition of AZC to HeLa cells did not induce any changes in cytosolic Ca^{2+}, prolonged treatment reduced the ER Ca^{2+} store content by about 25%. Interestingly, independently of its autophagy induction properties, resveratrol acutely decreased the ER Ca^{2+}-store content irrespectively of the presence or absence of IP_3Rs. Finally, we also investigated in various cellular models the role of the RyR3 isoform in autophagy. RyR3 overexpression impaired autophagic flux while the pharmacological inhibition of endogenously or ectopically expressed RyRs with dantrolene or ryanodine augmented the autophagic flux and the lysosomal turn-over.

These results underscore the importance of ER Ca^{2+}-release channels in the regulation of autophagy.

Impairment of Ca^{2+}-Dependent Inactivation of TRPC6 Mediated by Calmodulin Underlies Renal Channelopathy

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TRPC6 is a plasma membrane protein that in its tetrameric form is known to contribute to receptor-operated $Ca^{2+}/Na^{+}/$ influx in broad range of cells. The channel opening of TRPC6 is negatively regulated by Ca^{2+} bound Calmodulin (Ca^{2+}-CaM). Although CaM binding domain (CBD) in TRPC6 has been reported, mechanistic insight of CaM on Ca^{2+}-dependent inactivation (CDI) is still unveiled. Here we demonstrate that both lobes of CaM equally contribute to CDI, which is a very different result than CaM regulation on voltage-gated ion channels. To obtain mechanistic insights of inactivation, NMR and ITC titration experiments were carried out and both experiments resulted in 1:2 stoichiometry of Ca^{2+}CaM binding to TRPC6-CBD as well as over 100 times weaker affinity of N-lobe binding to CBD than C-lobe. These functional and biochemical results indicate that CDI can be explained by a close proximity of two TRPC6-CBDs due to a 'catch and close' mechanism by Ca^{2+}CaM. Furthermore, we found that coiled-coil segment located near the CBD domain of TRPC6 has a critical role for maintaining the proximal distance of two CBD's by its self-assembly, and deletion of the coiled-coil segment caused reasonable critical effect on CDI. Deletion or point mutations of the coiled-coil are implicated in focal segmental glomerulosclerosis. The coiled-coil mutants found in renal patients severely delayed CDI, thus would lead to critical damages in kidney cells.

Participation of Calcium Binding Protein 1 in Actin Dynamics and Phagocytosis of the Parasite Entamoeba Histolytica

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Entamoeba histolytica is a causative agent of amoebiasis which infects millions of people worldwide and is a major cause of morbidity and mortality in the developing countries. Amoebic motility and phagocytosis are vital cellular processes that determine virulence and pathogenesis of this parasite and blocking these process leads to an inhibition of cell proliferation and pathogenicity. Our group has identified a novel signaling pathway involved in phagocytosis of E. histolytica. Role of several kinases, calcium binding and other signaling proteins such as EhC2PK, EhAK1, EhCaBP1, EhCaBP3 and subunits of Arp2/3 complex has been investigated in relation to the phagocytosis. Calcium binding protein 1 (EhCaBP1) has been shown to regulate actin cytoskeleton by recruiting different effector proteins to the plasma membrane. However, detailed mechanism that regulates actin dynamics is not known. EhCaBP1 binds both G- and F-actin and thought to be involved in actin bundling. The interaction of EhCaBP1 with actin is specific as close homolog EhCaBP2 (with 79% sequence identity) does not have this property. Ca^{2+} binding defective mutant of EhCaBP1 showed reduced binding towards actin. Though this mutant reached phagocytic cups but over expression led to a defect in overall phagocytic rate. In order to understand the mechanism of interaction of EhCaBP1 with actin. A large number of alanine mutants were generated in the linker region of EhCaBP1 as linker deleted molecule could not bind actin. Both F- and G-actin binding assays of these mutants revealed a range of activities from no activity to complete abolition of activity (Ser71Ala). This mutant is being characterized with respect to its role in phagocytosis and interaction with other proteins, such as EhAK1.

Neuron-Astrocyte Communication in the Spinal Cord of Behaving Mice

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How do we recognize and perceive the rich sensory environment that surrounds us, or distinguish between hazardous and non-hazardous stimuli to guide our responses? Anatomical and physiological measurements indicate that our central nervous system (CNS) initiates these types of computations in the dorsal horn of the spinal cord. Different types of dorsal horn neurons play important roles in sensory and pain processing. In addition, non-neuronal cells such as glial cells play active roles in the pathogenesis and resolution of pain. However, despite the obvious importance of the spinal dorsal horn, we still know very little about how the activity patterns in the different cell types encode innocuous and noxious information, and how neurons and glial cells interact under healthy and diseased conditions. This has been partly due to a lack of technologies that allow measurement and manipulation of cellular activity patterns, particularly under awake behaving conditions. Using fluorescence imaging approaches we recently demonstrated that stable measurement of cellular population activity from the spinal dorsal horn of behaving mice is feasible. This enabled us to provide first insights into how sensory information from mechanoreceptors and nociceptors in the skin activates spinal neurons and glial cells in awake healthy mice, and how anesthesia powerfully suppresses their activity. Nevertheless, much remains to be learned about the cellular and computational logic of dorsal horn networks under healthy and diseased conditions. In this presentation I will describe some of our latest findings on the functional relationship between neuronal and glial cell activity in the spinal cord of behaving mice.

Monitoring Nanomolar Calcium with FLIM

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Neurons and glia are known to maintain low resting Ca^{2+} but its actual levels remain an enigma. These levels however are likely to determine the amount of endogenous Ca^{2+} buffers available locally, thus directly affecting the dynamics of rapid Ca^{2+} signals. We have therefore advanced an imaging technique that exploits high sensitivity of the OGB-1 fluorescence lifetime to free Ca^{2+}. The signal readout, which is independent of dye concentration, focus depth or light scattering, reliably reported [Ca^{2+}] between 5-200 nM. This was not affected by any major concomitants of cell function, such as [Mg^{2+}], pH, [Zn^{2+}], protein (actin) concentration, photobleaching, micro-viscosity, or temperature, within their physiological ranges. Equipped with this approach, we have found that whole-cell patch configuration has little effect on basal Ca^{2+} in dendrites of principal neurons, or in the astroglia connected to the patched cell via gap-junctions. Intracellular three-dimensional mapping of resting [Ca^{2+}] has revealed an unexpectedly reach, use-dependent information content carried by [Ca^{2+}] landscapes in neurons and astroglia. These data are unveiling some hitherto unexplored, potentially fundamental aspects of brain cell physiology pertinent to synaptic function. The ongoing experiments combine axonal [Ca^{2+}] imaging with single-quantum detection of glutamate release using an optical sensor.

Rewiring of Neuronal Networks by Astrocytic Ca^{2+} in the Somatosensory Cortex

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Peripheral neuropathic pain that includes mechanical allodynia remains poorly treated. While glial activation and altered nociceptive transmission within the spinal cord is associated with the pathogenesis of mechanical allodynia, changes in cortical circuits also accompanies peripheral nerve injury and may represent additional therapeutic targets. Dendritic spine plasticity in the S1 cortex appears within days following nerve injury, however, its causal relationship to allodynia and the underlying cellular mechanisms remain unsolved. Furthermore, whether glial activation occurs within the S1 cortex following injury and how it contributes to this S1 synaptic plasticity is unknown. Using in vivo two-photon imaging with genetic and pharmacological manipulations, we show that sciatic nerve ligation induces a re-emergence of immature mGluR5 signaling in S1 astroglia, which elicits spontaneous somatic Ca^{2+} transients, synaptogenic TSP-1 release and synapse formation. Such S1 astrocyte reactivation was evident only during the first week post-injury, correlating with the temporal changes in S1 extracellular glutamate levels and dendritic spine turnover. Blocking this astrocytic signaling pathway suppressed mechanical allodynia, while activating this pathway in the absence of any peripheral injury induced long-lasting (>1 month) allodynia. We conclude that reawakened astrocytes are a key trigger for S1 circuit rewiring and this contributes to neuropathic mechanical allodynia.

Parvalbumin Alters Mitochondrial Volume and ROS-Level in Oligodendrocyte Progenitor Cells and Mature Oligodendrocytes

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Forebrain glial cells - ependymal cells and astrocytes - acquire reactive phenotype upon injury-triggered parvalbumin (PV)-upregulation. Reactive oligodendroglia might also manifest regenerative properties similarly to reactive astrocytes. Based on the findings that free radicals, such as nitric oxide and reactive oxygen species (ROS) play a role in the pathogenesis of multiple sclerosis (MS), and on our own findings that PV-upregulation in glial cells is correlated with the level of oxidative stress, we postulated that PV-upregulation might protect oligodendrocytes against oxidative damage in MS. We overexpressed PV, resp. down-regulated it by shRNA using lentiviral transduction techniques in the rat oligodendrocyte progenitor cell (OPC) line CG-4. CG-4 cells remain in an undifferentiated state when exposed to proliferating media, and differentiate into mature oligodendrocytes when exposed to differentiation media. We examined the number and volume of mitochondria in OPCs and mature oligodendrocytes by live mitochondrial staining, and assessed oxidative stress level in live cells by the superoxide indicator dihydroethidium (DHE) reflecting the level of ROS. We found reduced mitochondrial number and volume in PV-overexpressing OPCs and mature oligodendrocytes, whereas increased mitochondrial number and volume in shPV-expressing ones. Furthermore, we found alterations in the levels of ROS.

Transcranial Direct Current Stimulation Triggers Cortical Metaplasticity Through Glial Calcium Elevation

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Global brain states regulate plasticity in cortical circuits but the underlying cellular and molecular mechanisms are unclear. In an attempt to optically BRC, strain ID: RBRC09650) that expresses the genetic Ca^{2+} indicator G-CaMP7 in astrocytes and a subpopulation of neurons (Monai et al. Nat Commun 2016). Using this mouse, we explored the cortical Ca^{2+} dynamics during transcranial direct current stimulation (tDCS), a treatment known to ameliorate various neurological conditions and enhance memory and cognition in humans. We found that tDCS induced large-amplitude astrocytic Ca^{2+} surges across the entire cortex with no obvious changes in cortical neuronal activity. Moreover, sensory evoked cortical responses were enhanced after tDCS, whereas this enhancement was not seen in a mouse model in which astrocytic Ca^{2+} surges are largely diminished (i.e. IP_3R_2-KO mice, Futatsugi et al. Science 2005), suggesting that tDCS alters the plasticity of the cortex (i.e. metaplasticity) through astrocytic Ca^{2+}/ IP_3 signaling. We suggest that this could be a prevailing mechanism of action of tDCS (Monai et al. Neurogenesis 2016). We are currently investigating if tDCS affects other glial cell types.

Ion Channels and Promotion of Cell Death Pathways in Triple Negative Breast Cancer Cells

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Triple negative breast cancers lack the expression of targets for hormonal and molecular targeted therapies. Triple negative breast cancers have a significant overlap with the basal molecular subtype which are associated with a poor prognosis and an increased occurrence of metastasis, particularly to the brain. Some basal breast cancers appear to differentially express specific calcium permeable ion channels. Pharmacological modulation of these ion channels can have pronounced effects on the viability of basal-like breast cancer cell lines though the induction of specific cell death pathways.

The SERCA Interactors Calnexin and TMX1 Regulate Mitochondria Metabolism

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Tumor cell metabolism is frequently aberrant and shifts its energy generation away from mitochondria towards glycolysis. Currently, few molecular mechanisms are known that can lead to such a metabolic shift. Research from several labs, including ours, have identified calcium-mediated crosstalk at the mitochondria-associated membrane (MAM) as such a mechanism. The MAM is formed by close apposition between the endoplasmic reticulum (ER) and mitochondria, where the two organelles exchange calcium ions. This flux is a determinant of mitochondria metabolism, and depends on the following factors: i. The extent of ER-mitochondria apposition, ii. The extent of mitochondrial calcium release and uptake. iii. The extent of ER calcium release and uptake. Our lab studies proteins that control this latter factor. We have identified ER chaperones and oxidoreductases as critical regulator of ER-mitochondria calcium flux. These proteins exert their functions as regulators of ER calcium channels and pumps. The presentation will focus on the latest results on calnexin, an activator of SERCA pumps. We show that calnexin determines mitochondria metabolism through this function, as an opposing mechanism to the previously identified inhibition of SERCA by TMX1. Moreover, calnexin and TMX1 aberrant expression patterns in cancer, consistent with their molecular functions.

Regulation of the CaMKKβ/AMPK Signaling Cascade by Phosphorylation

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Ca^{2+}/calmodulin-dependent protein kinase kinases (CaMKKs) were originally identified as a member of the calmodulin kinase (CaMK) family, which phosphorylate and activate two multifunctional CaMKs including CaMKI and CaMKIV, constituting Ca^{2+}-dependent kinase cascades named CaMK cascades. CaMKK in mammals is derived from two genes (*CaMKKα* and *CaMKKβ*), with approximately 70% of the amino acid sequence homology in the catalytic domains [1,2]. Enzymatic activity of CaMKK is regulated by the autoinhibitory mechanism and induced by Ca^{2+}/CaM-binding in a similar manner to other CaMKs [3]. Except for the downstream CaMKs, 5'-AMP-activated protein kinase (AMPK) has been identified as a novel target kinase for CaMKKβ but not for CaMKKα. We recently demonstrated that a single amino acid residue in the subdomain VIII (Leu358 in CaMKKβ/Ile322 in CaMKKα confers, at least in part, a distinct recognition of AMPK [4]. Recent studies using genetical and pharmacological approaches using a CaMKK inhibitor (STO-609) [5] have demonstrated that the CaMKKβ/AMPK pathway involves in various Ca^{2+}-dependent AMPK-mediated signal transduction processes including appetite control, metabolic regulation, autophagy, and cancer growth [2]. Whereas CaMKK phosphorylates downstream protein kinases in multiple signaling cascades, the accumulated evidence indicates that CaMKK itself is regulated by PKA-mediated, CDK5/GSK3-mediated phosphorylation [6] and autophosphorylation [7]. In this report, we present a novel phosphorylation-dependent regulatory mechanism of CaMKKβ/AMPK signaling cascade *in vitro* as well as *in vivo*.

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Septin 7 Binding to Butyrate-Responsive Elements in the CALB2 Promoter Negatively Regulates Calretinin Expression in Malignant Mesothelioma

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The calcium-binding protein calretinin (gene: CALB2) is to date the most sensitive and specific marker for the diagnosis of malignant mesothelioma (MM). MM is a very aggressive tumor strongly linked to asbestos exposure and with no existing cure so far. The mechanism of calretinin regulation, as well as its distinct function in MM is still poorly understood. Here, DNA-binding assays followed by peptide shotgun-mass spectroscopy analysis revealed septin 7 as a butyrate-dependent transcription factor binding to a CALB2 promoter region containing previously identified butyrate-responsive elements (BRE) resulting in decreased calretinin expression. Accordingly, lentiviral-mediated septin 7 overexpression decreased calretinin expression levels in MM cells. Unexpectedly, the regulation was found to operate bi-directionally, i.e. calretinin overexpression decreased septin 7 levels. During murine embryonic development calretinin and septin 7 were found to be co-expressed in embryonic mesenchyme and developing mesothelial cells. In MM cells, during cytokinesis calretinin and septin 7 not only colocalized to distinct regions within the cells, but interacted as evidenced by co-immunoprecipitation experiments. The two proteins localized to distinct regions of the cleavage furrow and in the midbody region. Our results demonstrate septin 7 not only serving as a “cytoskeletal” protein, but also as a transcription factor repressing calretinin expression. The negative regulation of calretinin by septin 7 and vice versa sheds new light on mechanisms possibly implicated in MM formation and identifies these proteins as transcription regulators and putative targets for MM therapy.

Structural Basis and Physiological Significance of Ryanodine Receptor Luminal Calcium Activation

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Cardiac myocyte contraction is driven by the coordinated release of Ca^{2+} from the sarcoplasmic reticulum (SR). This release of Ca^{2+} occurs through the cardiac ryanodine receptor (RyR2). Physiologically, Ca^{2+} release occurs in response to an influx of Ca^{2+} through L-type Ca^{2+} channels, via a mechanism termed Ca^{2+} induced Ca^{2+} release (CICR). This transient increase in cytosolic Ca^{2+} activates the RyR2 channel as a result of Ca^{2+} binding to the cytosolic Ca^{2+} activation site of the channel. However, RyR2 is also known to be activated in the absence of cytosolic Ca^{2+} elevation, under conditions of SR Ca^{2+} overload. This phenomenon is commonly known as spontaneous Ca^{2+} release, or store overload induced Ca^{2+} release (SOICR), due to its dependence on SR Ca^{2+} load. SOICR occurs when SR Ca^{2+} content exceeds a threshold level, which can be due to either an increase in SR Ca^{2+} concentration above the threshold or a reduction in the threshold to a level below the SR Ca^{2+} concentration. Although SOICR has been well characterized as an underlying mechanism of multiple pathologies, exactly how RyR2 detects and responds to SR luminal Ca^{2+} has yet to be defined. Here I will talk about the structural basis of SR luminal Ca^{2+} activation of RyR2 in light of recent three-dimensional structures of RyRs, and discuss the pathological significance of RyR2 luminal Ca^{2+} activation.

Calcium-Dependent Regulation of Human Cardiac Sodium Channel Inactivation by Calmodulin

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The human cardiac sodium channel ($Na_V1.5$) is essential for the generation and propagation of action potentials in the heart. Previous studies have demonstrated that $Na_V1.5$ is sensitive to cytosolic Ca^{2+} and interacts with the Ca^{2+}-sensor calmodulin (CaM), but the underlying mechanism(s) are complex, controversial and remain incompletely defined. We have carefully re-examined binding of CaM to the intracellular loop (i.e. the linker between membrane spanning domains III and IV) that is critical for inactivation of $Na_V1.5$, which is termed the inactivation gate (IG). Combining NMR, X-ray crystallography, and small angle X-ray scattering, we generated a structure of CaM engaged on the IG. We found that when presented with the complete IG, CaM binds in an extended mode to the two distinct CaM binding motifs that had each been previously studied independently. Inserting this structure into a homology model of the full-length channel suggests a mechanism for how CaM interacts with the IG and affects channel inactivation. To begin to test this proposal, whole cell patch clamp recordings on $Na_V1.5$ mutants were performed at fixed [Ca^{2+}], which enabled probing of the specific effects of the CaM-IG interaction without altering other Ca^{2+} sensitive properties of the channel. The data reveal that the CaM-IG interaction specifically affects channel function by promoting recovery from inactivation. Our results identify a strong CaM-IG interaction that forms the basis for Ca^{2+} regulation of $Na_V1.5$ channel inactivation, and suggest a new potential mechanism for the cause of life threatening arrhythmias.

Genetic and Biophysical Basis of Calmodulinopathy, and Functional Rescue by Genome-Editing in Patient-Derived iPS Cardiomyocytes

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Calmodulin (CaM) is a ubiquitous calcium sensor molecule encoded by three genes *CALM1-3* with an identical amino acid sequence. Mutations in *CALM1-3* have been associated with severe forms of life-threatening arrhythmias, such as long QT syndrome (LQTS) and catecholaminergic polymorphic ventricular tachycardia (CPVT). By using conventional and whole-exome sequencing in genotype-negative LQTS, we identified 6 novel de novo missense mutations (one in *CALM1* and 5 in *CALM2*) in 4 subjects with LQTS (N98S, N98I, E105A, D134H) and 2 subjects with clinical features of both LQTS and CPVT (D132E, Q136P). Age of onset of life-threatening events ranged from 1 to 9 years. Mutations affect conserved residues located within Ca^{2+}-binding loops III (N98S, N98I, E105A) or IV (D132E, D134H, Q136P) and caused reduced Ca^{2+}-binding affinity. To determine underlying mechanism through which heterozygous calmodulin mutations lead to severe LQTS, we established induced pluripotent stem cells (hiPSCs) from a 12-year-old boy with LQTS carrying a *CALM2*-N98S, and assessed mutant allele-specific knockout using a CRISPR-Cas9 genome editing technology for the treatment of LQTS. The hiPSCs lines with or without genome editing were differentiated into cardiomyocytes (CMs), and action potentials and L-type Ca^{2+} channel (LTCC) currents were analyzed by the patch-clamp technique and compared with those of healthy control. CMs of *CALM2*-N98S exhibited significantly lower beating rates, prolonged action potential durations, and impaired inactivation of LTCC currents compared with control cells. Notably, ablation of the mutant allele rescued the electrophysiological abnormalities of CMs with *CALM2*-N98S, indicating that the mutant allele caused dominant-negative suppression of LTCC inactivation, resulting in prolonged action potential duration. In conclusion, we successfully recapitulated the disease phenotypes of calmodulinopathy and revealed that inactivation of LTCC currents was impaired in *CALM2*-N98S hiPSC model. Furthermore, allele-specific ablation using the latest genome-editing technology provided important insights into a promising therapeutic approach for inherited cardiac diseases.

Axial Tubule Junctions Control Rapid Ca^{2+} Release and Induce Maladaptive Electromechanical Remodeling in Atria

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Atrial myocytes (AM) are characterized by slow intracellular Ca^{2+} signaling and sparse transverse tubule (TT) invaginations. In contrast, contractile activation of healthy atrial muscle is rapid, yet prone to loss-of-function and arrhythmogenic remodeling by maladaptive hypertrophy.

OBJECTIVES: 1) overcome the slow Ca^{2+} signaling versus rapid contractile activation paradox; and 2) elucidate atrial mechanisms of contractile and arrhythmogenic electrical dysfunction during hypertrophic remodeling.

RESULTS: We identified cell-specific large intracellular membrane structures in mouse AM, termed axial tubules (AT), connected to the surface membrane through sparse TTs. Electrical stimulation rapidly activated central Ca^{2+} release through extensive AT junctions with the sarcoplasmic reticulum via Ryanodine Receptor (RyR2) Ca^{2+} release channels. Similar AT structures and elongated RyR2 clusters were confirmed in human atrial tissue. Unexpectedly, intracellular onset of AT-dependent Ca^{2+} release occured significantly faster compared to the surface membrane. This correlated with increased phosphorylation of junctional RyR2 clusters and earlier sarcomere shortening, in contrast with non-junctional least phosphorylated RyR2 clusters. Accordingly, knockin mice expressing phosphorylation incompetent RyR2 channels displayed both significantly depressed sarcomere shortening and atrial function. Importantly, left atrial hypertrophy 4 weeks after aortic banding (post-TAC) induced profound AT proliferation, increasing the fraction of highly phosphorylated RyR2 clusters, which accelerated Ca^{2+} release despite decreased RyR2 protein expression. Finally, atrial mapping of action potential (AP) propagation post-TAC showed significant conduction velocity slowing and AP prolongation.

SUMMARY: Compartmentalized phosphorylation of RyR2 channels in AT junctions facilitates rapid intracellular Ca^{2+} release and contractile activation in AM. In contrast, hypertrophic remodeling promotes contractile dysfunction and latent arrhythmogenic substrates. While AP shortening was described during atrial fibrillation, altered Ca^{2+} release during early hypertrophic remodeling with conduction slowing indicates distinct proximal disease mechanisms.

Functional Molecular Complexes of Junctophilin-2 and Caveolin-1 are Essential for Ca^{2+} Microdomain Formation in Vascular Smooth Muscle Cells

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Functional coupling between large-conductance Ca^{2+}-activated K^+ channels (BK_{Ca}) in plasma membrane (PM) and ryanodine receptors (RyRs) in sarcoplasmic reticulum (SR) is an essential mechanism for mechanical force regulation in smooth muscle cells (SMCs). Spontaneous Ca^{2+} release through RyRs (knows as Ca^{2+} sparks) and subsequent BK_{Ca} activation detected as spontaneous transient outward currents (STOCs) occur within PM-SR junctional sites, often called Ca^{2+} microdomains. We have previously reported that Caveolae, Ω-shaped invaginations of PM, accumulate BK_{Ca} within the structure and enhance coupling efficiency between Ca^{2+} sparks and BK_{Ca}/STOC activity in mouse mesenteric artery SMCs (mMASMCs). However, the molecular basis between caveolae and RyRs in SR membrane is unclear. In the present study, we demonstrated that both caveolin-1 (Cav1), a caveola forming protein, and junctophilin-2 (JP2), a bridging protein between PM and SR, reciprocally contribute to Ca^{2+} microdomain formation. Co-immunoprecipitation using rat mesenteric arterial tissues and double-immunocytochemical staining analyses by total internal reflection fluorescent microscopy revealed the direct interaction between JP2 and Cav1. Knockdown of JP2 in mMASMCs significantly decreased the co-localization of Cav1 and RyRs and the amplitude of STOCs. The contraction induced by the inhibition of BK_{Ca}/STOC activity and subsequent membrane depolarization in siJP2-treated mMASMCs was significantly smaller than that in siControl. These results suggested that the novel interaction of JP2 with Cav1 seems to provide a structural/functional basis for Ca^{2+}-mediated crosstalk between RyRs and BK_{Ca}, and likely positions the PM-SR junctional sites to convert Ca^{2+} spark signals into hyperpolarizing signals in SMCs lacking transverse tubular system.

Calcium-Dependent Signaling in Dendritic Spines

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Activity-dependent changes in synaptic strength and structure are believed to be cellular basis of learning and memory. A cascade of biochemical reaction in dendritic spines, tiny postsynaptic compartments emanating from dendritic surface, underlies diverse forms of synaptic plasticity. The reaction in dendritic spines is mediated via signaling networks consist of hundreds of species of proteins. We have developed unique optical techniques to elucidate the operation principles of such signaling networks. First, based on 2-photon fluorescence lifetime imaging and highly sensitive biosensors, we have developed techniques to image signaling activity in single dendritic spines. We have succeeded in monitoring activity of several key signaling proteins in single spines undergoing structural and functional plasticity. This provided new insights into how the spatiotemporal dynamics of signaling are organized during synaptic plasticity. We have developed sensitive and specific sensors for CaMKI, CaMKII, Rho GTPase proteins, Rab GTPase proteins, protein kinase C isozymes (α, β, γ etc) and the BDNF receptor TrkB. By monitoring signaling components with high spatiotemporal resolution, we expect to reveal the mechanisms underlying the spatiotemporal regulation of signaling dynamics underlying synaptic plasticity and learning and memory.

The NMDA Receptor Calcium Signaling Paradox: Neuroprotection Versus Death Signaling

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The dialogue between the synapse and the nucleus controls activity-driven gene transcription and is vital for virtually all adaptive responses in the nervous system including the build-up of a neuroprotective shield, the formation of memories, but also unwanted adaptations such a chronic pain or addiction. Calcium signals generated by synaptic activity and the opening of synaptic NMDA receptors and voltage-gated calcium channels serve as initiators of this communication pathway. They also mediate the propagation along the synapse-to-nucleus axis, although additional protein-based transport processes, such as the ERK-MAP kinase cascade, play a role (Hagenston and Bading, 2011). Nuclear calcium transients represent an important signaling endpoint in synapse-to-nucleus communication and function as master switch for adaptations-associated transcription. Blockade of nuclear calcium signaling in hippocampal neurons eliminates 'acquired neuroprotection', an activity-driven form of adaptation in which neurons that have been electrically activated are more resistant to harmful, cell death-inducing conditions. Similarly, the consolidation of memories and their extinction, as well as the development of chronic pain in mice is critically dependent on nuclear calcium signaling (Bading, 2013). In my presentation I will outline the features of the synapse-to-nucleus communication axis, discuss its genomic targets, and summarize how in neurodegenerative conditions this transcription-promoting axis is being antagonized by a cell death promoting signaling pathway activated by extrasynaptic NMDA receptors (Hardingham and Bading, 2010; Bading, 2017).

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Neuronal Store Operated Calcium Entry as Novel Therapetic Target for Treatment of Alzheimer's Disease

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Memory loss in Alzheimer's disease (AD) results from “synaptic failure”. Mushroom dendritic spine structures are essential for memory storage and the loss of mushroom spines may explain memory defects in aging and AD. To understand the basis for memory loss in AD we performed a series of mechanistic studies of hippocampal synaptic spines in mouse models of familial AD (FAD). In our experiments we used presenilin 1 (PS1) M146V knockin (PS1KI) and APPKI models of FAD. In the course of these studies we discovered an existence of spine maintenance pathway that is mediated by neuronal store-operated Ca^{2+} entry (nSOC) in postsynaptic spines (Sun et al, 2014). We established that nSOC pathway plays a key role in stability of mushroom spines by constitutively activating synaptic CaMKII kinase. We further demonstrated that synaptic nSOC is controlled by stromal interaction molecule 2 (STIM2) and that STIM2-nSOC-CaMKII pathway is compromised in PS1KI and APPKI neurons, in aging neurons and in sporadic AD brains due to downregulation of STIM2 protein (Sun at al, 2014; Zhang at al 2015). Moreover, we have demonstrated that expression of STIM2 protein rescues synaptic nSOC and mushroom spine loss in PS1KI and APPKI hippocampal neurons (Sun at al, 2014; Zhang at al 2015) and protects mushroom spines from amyloid synaptotoxicity (Popugaeva at al, 2015). In recent studies we determined that STIM2-gated nSOC channels are composed of TRPC6 and Orai2 subunits (Zhang at al, 2016). We identified a novel molecule NSN21778 that can stimulate activity of nSOC pathway in the spines and rescue mushroom spine loss in both presenilin and APP knock-in mouse models of AD. We further demonstrated that NSN21778 rescue hippocampal long-term potentiation impairment in APP knock-in mouse model. We conclude that STIM2-regulated TRPC6/Orai2 nSOC channel complex in dendritic mushroom spines is a new therapeutic target for treatment of memory loss in aging and AD and that NSN21778 is a potential candidate molecule for therapeutic intervention in brain aging and AD.

Calmodulin Inhibition of IP_3 Receptor Mediated Calcium Release

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Inositol 1,4,5-trisphosphate (IP_3) receptors (IP_3Rs) function as gate-keepers for calcium (Ca^{2+}) release from internal stores into the cytosol in a variety of cell types. Even though they are found in multiple intracellular membranes, the endoplasmic reticulum (ER) remains the primary site of IP_3-induced Ca^{2+} release. IP_3Rs are regulated not only by IP_3, but also by Ca^{2+} itself. Evidence suggests that IP_3 must bind to the receptor in order to sensitize it to Ca^{2+}. The IP_3R response to cytosolic Ca^{2+} is biphasic: at lower levels, Ca^{2+} activates the receptor whereas it inhibits it at higher levels. While the site triggering IP_3R opening is believed to be situated in the receptor itself, the site triggering Ca^{2+} mediated channel closing remains uncharacterized, and this process may be indirectly controlled through an accessory protein. The Ca^{2+}-binding protein calmodulin (CaM) is well-known for its ability to modulate Ca^{2+}-transporting proteins, such as plasma membrane Ca^{2+}-channels and SR ryanodine receptors. Indeed, CaM has also long been known to exert an inhibitory effect on IP_3R Ca^{2+} release. Nevertheless, details about the overall regulation mechanism remain elusive. Curiously, CaM has been reported to modulate IP_3Rs both in a Ca^{2+}-dependent and ?independent manner. Here, we investigated the Ca^{2+}- and IP_3-dependence of CaM regulation of IP_3R1. Moreover, we assessed the influence of engineered as well as human disease-causing CaM mutations on IP_3R1 function.

Mitochondrial Calcium Signalling in Cell Life and Death

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Mitochondrial Ca^{2+} uptake regulates a wide array of cell functions, from stimulation of aerobic metabolism and ATP production in physiological settings, to induction of cell death in pathological conditions. The molecular identity of the Mitochondrial Calcium Uniporter (MCU), the highly selective channel responsible for Ca^{2+} entry through the IMM, has been described six years ago. Since then, research has been conducted to clarify the modulation of its activity, which relies on the dynamic interaction with regulatory proteins, and its contribution to the pathophysiology of organs and tissues. The Ca^{2+} permeant pore comprises three proteins: MCU, MCUb (the dominant-negative subunit that incorporates into the uniporter channel and reduces its activity), and EMRE (a 10 kDa protein that seems important for MCU channel activity and also for mediating the interaction between the MCU and the regulatory subunits MICU1 and MICU2). The Ca^{2+}-binding EF-hand-containing proteins MICU1, MICU2, MICU3 and MICU1.1 provide the complex with a highly sophisticated gatekeeping mechanism that keeps the channel closed at low $[Ca^{2+}]_{cyt}$ (resting conditions) and activates it upon cell stimulation thus explaining one of the peculiar properties of mitochondrial Ca^{2+} uptake, i.e., the sigmoidal response to $[Ca^{2+}]$cyt. How the molecular complexity of the uniporter is linked to its physiological role is discussed. Particular attention has been placed on characterizing the molecular structure and regulation of the MCU complex in addition to its pathophysiological role, with a focus on striated muscle tissues, cancer and neurodegeneration.

MICU2 Spatially Restricts Ca^{2+} Crosstalk Between IP_3R and MCU Channels by Regulating Threshold and Gain of MICU1-Mediated Inhibition and Activation

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Calcium (Ca^{2+}+) uptake by mitochondria plays important roles in regulation of bioenergetics, programmed cell death and cytoplasmic Ca^{2+}+ signaling. The large inner membrane voltage generated by the respiratory chain provides a tremendous driving force for Ca^{2+}+ uptake from cytoplasm into the mitochondrial matrix. Nevertheless, mitochondrial matrix Ca^{2+}+ must be tightly controlled to maintain a balance between requirements for optimal energy production and toxic effects of Ca^{2+}+, including activation of the permeability transition pore. The major mechanism for Ca^{2+}+ entry into mitochondria is the Ca^{2+}+ uniporter, shown to be a highly-selective Ca^{2+}+ channel with high carrying-capacity. The uniporter is a complex containing MCU, the pore forming subunit, and other proteins, including EF-hand domaincontaining MICU1 and its paralogs MICU2 and MICU3. The roles of MICU proteins in regulating MCU channel activity are controversial. We employed HEK-293T cells with MICU1 or MICU2 genetically deleted to address the molecular mechanisms by which MICU1 and MICU2 regulate MCU function, particularly in the previously uncharacterized [Ca^{2+}+]c regime below ~1 μM. By characterizing the roles of Ca^{2+}+-sensing domains in MICU1 and MICU2 on MCU activity at both low (0.1-1 μM) and high (1-10 μM) [Ca^{2+}+] cyt, we have identified the individual roles of each protein in coupling changes in [Ca^{2+}+]cyt to MCU regulation over a range of [Ca^{2+}+]cyt experienced by mitochondria. Our results suggest that MICU1 alone can mediate so-called gatekeeping, inhibition of channel open probability at low [Ca^{2+}+]cyt, as well as highly cooperative activation of MCU channel activity by higher [Ca^{2+}+]cyt, whereas the fundamental role of MICU2 is to regulate the threshold and gain of MICU1- mediated inhibition and activation of MCU. Our results provide a unifying model for the role of the MICU1/2 hetero-dimer in MCU channel regulation and they suggest an evolutionary role for MICU2 to spatially restrict Ca^{2+}+ crosstalk between single InsP3R and MCU channels.

Ca^{2+} Sensing by MICU Proteins for Mitochondrial Ca^{2+} Uptake

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Early studies of mitochondria isolated from various tissues have demonstrated that Ca^{2+} uptake is driven by the membrane potential, and is mediated by a ruthenium red-sensitive electrogenic uniport, referred as "Ca^{2+} uniporter" (mtCU). Electrophysiological recording of mtCU documented a similar inwardly rectifying Ca^{2+} current in mitoplasts derived from different tissues but great differences appeared in the current density, which was particularly low in cardiac mitochondria. Recently, the major mtCU forming proteins have been identified, including the pore, MCU, its dominant-negative form, MCUb, a scaffold, EMRE, and Ca^{2+}-sensitive regulators, MICU1 and MICU2. To date, a MICU complex (a hetero/homo-dimer of MICU1 and MICU2) appears to determine both the threshold and cooperative activation of the mtCU by Ca^{2+}, thus providing a mechanism for the sigmoidal [Ca^{2+}] dependence of the mtCU. MICU1 deletion in mouse is perinatal lethal, likely because of mitochondrial Ca^{2+} overload-induced injury. mtCU components show tissue-specific expression and MICU1 is expressed at a low level in striated muscle. The tissue specific differences in the mtCU current and molecular composition are particularly interesting in the context of the distinct calcium signaling patterns that mitochondria from different tissues have to cope with. We have created organ-specific knockouts of MICU1 in hepatocytes, skeletal muscle fibers and neurons to determine the tissue specific physiological functions of MICU1, which studies will be shown in this presentation.

All Roads Lead to Calmodulin

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In memory of Dr. Claude B. Klee, the National Institutes of Health, Bethesda MD.

Calmodulin (CaM) is a multifunctional, calcium-binding, signal transduction protein expressed in all eukaryotic cells. It is an essential intracellular receptor of the secondary messenger Ca^{2+}, and upon binding of Ca^{2+}, CaM becomes active in transmitting the calcium signal to a wide variety of enzymes and proteins such as kinases, phosphatases, and transmembrane proteins. CaM is therefore considered a Ca^{2+} sensor protein, in contrast to Ca^{2+} buffer proteins, whose main functions are to store and sequester Ca^{2+} in specific cellular locations. CaM and other Ca^{2+} sensor and buffer proteins all use the so-called 'EF-hand', which was first discovered in parvalbumin. The EF-hand consists of two helices representing the thumb and index finger, which coordinate a calcium ion in the palm of a hand-like structure. CaM is the founding member of the EF-hand superfamily of Ca^{2+} binding proteins, which now consists of more than 60 subfamilies including calcineurin, troponin, calbindins, S100s, calpains, NCSs, recoverin, and STIMs. The mechanisms of Ca^{2+}/CaM-dependent activation of target proteins have been of major interest in the field and extensive studies over the past decades have revealed tremendous molecular diversity in the fundamental mechanisms of CaM target recognition and activation (BBRC. 2015, 460:5-21; Cell. 2002,108:739-42; Trends Biochem Sci. 1996, 21:14-7; Annu Rev Biophys Biomol Struct. 1995, 24:85-116). Interestingly, a large number of the aforementioned EF-hand proteins, which have also been well-studied over the years, follow the initial paradigm of Ca^{2+}/CaM-dependent target activation, yet many studies have revealed surprising variations and new ways of modulating downstream signaling cascades in a Ca^{2+}-dependent manner. Re-reviewing some historical investigations on Ca^{2+}/CaM activation of protein kinases such as myosin light chain kinase and CaM kinase II, I will discuss the themes and variations established to date for Ca^{2+}-dependent target regulation by the CaM superfamily. (Supported by CIHR, NSERC, HSFC, CFI, PMCRF)

Direct Measurement of the Strength of Microtubule Attachment to Yeast Centrosomes

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Centrosomes, or spindle pole bodies (SPBs) in yeast, are vital mechanical hubs that maintain load-bearing attachments to microtubules during mitotic spindle assembly, spindle positioning, and chromosome segregation. However, the strength of microtubule-centrosome attachments is unknown, and the possibility that mechanical force might regulate centrosome function has scarcely been explored. To uncover how centrosomes sustain and regulate force, we purified SPBs from budding yeast and used laser trapping to manipulate single attached microtubules in vitro. Our experiments reveal that SPB$_s$microtubule attachments are extraordinarily strong, rupturing at forces approximately fourfold higher than kinetochore attachments under identical loading conditions. Furthermore, removal of the calmodulin-binding site from the SPB component Spc110 weakens SPB$_s$microtubule attachment in vitro and sensitizes cells to increased SPB stress in vivo. These observations show that calmodulin binding contributes to SPB mechanical integrity and suggest that its removal may cause pole delamination and mitotic failure when spindle forces are elevated. We propose that the very high strength of SPB$_s$microtubule attachments may be important for spindle integrity in mitotic cells so that tensile forces generated at kinetochores do not cause microtubule detachment and delamination at SPBs.

The Unique Tail of CNAβ1, a Non-Canonical Calcineurin A Isoform, Confers Novel Regulatory Properties and Promotes Membrane Localization

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Calcineurin, the conserved Ca^{2+}/calmodulin-regulated phosphatase and target of immunosuppressants, plays important roles in the circulatory, nervous and immune systems. Calcineurin activity depends strictly on Ca^{2+} and Ca^{2+}/ calmodulin to relieve autoinhibition of the catalytic subunit (CNA) by its C-terminus. The C-terminus encodes a calmodulin-binding domain (CBD) and two inhibitory sequences: AID (autoinhibitory domain), and AIS (autoinhibitory segment), which block the catalytic center and a conserved substrate-binding groove, respectively. However, CNAβ1, an atypical catalytic subunit isoform generated by alternative 3' end processing, contains a divergent C-terminus that lacks the AID. Biochemical characterization of CNAβ1, which is conserved in vertebrates and ubiquitously expressed, identified a distinct C-terminal autoinhibitory sequence in the CNAβ1 tail, LAVP, which blocks the substrate-binding groove. Thus, the CNβ1 holoenzyme is autoinhibited at a single site by two inhibitory sequences, the AIS and LAVP, which compete with each other and substrates for the substrate-binding groove. This novel regulatory strategy confers unique enzymatic properties to CNβ1, whose physiological substrates are unknown.

The CNβ1 C-terminal tail also contains conserved, palmitoylated, cysteine residues that promote CNβ1 association with membranes, in contrast to the canonical CNβ2 isoform, which is predominantly cytosolic. Affinity-purification mass spectrometry identified CNβ1-specific interactors, many of which are membrane proteins. These include a complex that targets PI4KIIIα to the plasma membrane, where it synthesizes phosphatidylinositol-4-phosphate (PI4P), a precursor of the critical signaling phospholipid, $PI(4,5)P_2$. CNβ1 is enriched in PI4P-containing membrane domains, where it may regulate PI4P synthesis to provide a Ca^{2+}-dependent mechanism for replenishment of PI4P during signaling.

Revisiting the Pharmacology of Calmodulin

Jacques Haiech

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Claude Klee has built her career on the study of calmodulin and several of its targets. She did not inherit this topic from her thesis, neither from her postdoc supervisor, but built her scientific journey in the calmodulin field in an original and very personal way.

In this presentation, we will focus on the analysis of calmodulin and on the emergence of a calmodulin-calcium-target protein code that allows the construction of a specific pharmacology. The presentation will not present a complete story, but a path of research that was opened by Claude Klee in the early 1970s.

In the 70-80s: Most of the scientific community is considering calmodulin as a cofactor without too much personality. The recognition is the same for all targets. A mutation is neutral or lethal. Calcium binding sites are independent and equivalent.

From 1985 to the 1990s: Calmodulin is shown to possess interacting and non-equivalent calcium sites. A battle is going to be played between the allosteric model of Monod, Wyman, Changeux and the induced fit model of Koshland.

Calmodulin has a specific recognition language with its targets. Most calmodulin mutations are neither lethal nor neutral, but affect a subset of interactions between calmodulin and its targets.

In the 2000s, the complete mechanism of interaction between calmodulin/calcium/target proteins is being revisited. A starting schema was presented by Claude Klee, Jacques Demaille and myself in 1981 (ionic and spatio-temporal regulation: the calcisome concept). Further work performed in the last two decades refined the model.

A specific pharmacology of calmodulin is emerging. Calmodulin has become a multiplexer of the calcium signal with a specific code of interaction with its partners that can be perturbed with small molecules. We are at the beginning of the analysis of the calmodulin code and its specific pharmacology.

Expanding the Range of Affinities and Colours in the Toolbox of Genetically Encoded Ca^{2+} Indicators

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Fluorescent protein-based Ca^{2+}+ indicators have revolutionized our ability to visualize the otherwise invisible world of intracellular signalling. Genetically encoded Ca^{2+}+ indicators are FP-containing proteins that change their fluorescence intensity or color in response to a change in Ca^{2+}+ concentration. These indicators are frequently used in combination with optogenetic actuators to enable simultaneous control and visualization of cellular signalling with precise spatial and temporal resolution. In this seminar I will describe our most recent efforts to use protein engineering to make a new generation of genetically encoded Ca^{2+}+ indicators with improved properties and an expanded range of potential applications. Specifically, I will present our efforts to engineer red and near-infrared Ca^{2+}+ indicators that are suitable for use in combination with blue-light activatable optogenetic actuators. In addition, I will discuss our efforts to modify the affinity of various indicators to optimize them for use in environments other than the cytoplasm.

Fluorescent/Bioluminescent Protein-Based Ca^{2+} Probes and Photo Manipulation for Imaging of Physiological Functions

Tomoki Matsuda, Takeharu Nagai

The Institute of Scientific and Industrial Research, Osaka University, Japan

Calcium is a universal signalling factor for biological phenomena thus probes to visualize Ca^{2+} directly in cells or organisms have been actively developed. Fluorescent protein based Ca^{2+} probes in particular have made remarkable developments. From the probes using Calmodulin as a sensing domain, many new Ca^{2+} probes have been iteratively developed and nowadays reports of spatiotemporal resolution of biological phenomena in living systems are commonplace. Key developments were enhancement of fluorescence intensity and dynamic range to improve signal-to-noise ratio. In addition, sensitivity has been improved has been pursued towards resolution of single-spike activity in neurons. At the same time, developments in other directions, in particular Ca^{2+} affinity allow observation of Ca^{2+} dynamics in the various environments. For example, low affinity Ca^{2+} probes can visualize the Ca^{2+} dynamics in high Ca^{2+} concentration environments like the ER. Here we continue the theme of Ca^{2+} probe diversification by using photoswitchable fluorescent proteins that change their fluorescence state by photostimulation. We also introduce the bioluminescent Ca^{2+} imaging which has the fundamental benefits (no photobleaching and independence from autofluorescence) with increased compatibility with optogenetic control.

Optogenetic Control of Calcium Signaling in Cells and Animals

Won Do Heo

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My group has been developing various optogenetic tools for the study of cell signaling in live cells as well as neuronal functions in vivo. In this talk I will talk about two optogenetic tools we have developed to control endogenous calcium channels by exploiting plant blue light receptor cryptochrome 2. OptoSTIM1 is an optogenetic tool for manipulating intracellular Ca^{2+} levels through activation of Ca^{2+}-selective endogenous Ca^{2+} release-activated Ca^{2+} (CRAC) channels. Using OptoSTIM1, we quantitatively and qualitatively controlled intracellular Ca^{2+} levels in various biological systems, including zebrafish embryos and human embryonic stem cells. We demonstrated that activating OptoSTIM1 in the CA1 hippocampal region of mice selectively reinforced contextual memory formation. FGFR (optoFGFR1) is an optically controlled Receptor Tyrosine Kinase which activate intracellular FGF signaling. Using optoFGFR1, we elucidated the spatial role of local Ca^{2+} signals in directed cell migration. We revealed the existence of Ca^{2+} sparklets mediated by L-type voltage-dependent Ca^{2+} channels in the rear part of migrating cells. Notably, we found that this locally concentrated Ca^{2+} influx acts as an essential transducer in establishing a global front-to-rear increasing Ca^{2+} gradient. This asymmetrical Ca^{2+} gradient is crucial for maintaining front-rear morphological polarity by restricting spontaneous lamellipodia formation in the rear part of migrating cells. Collectively, our new optogenetic tools are very useful for the study of spatial and temporal roles of calcium signaling in cells and animals.

Novel Localized and Transient RyR1 Junctional Calcium Responses and Dynamics by Calcium Sensors, CatchER$^+$ and CatchER$^+$-JP45

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The precise spatio-temporal characteristics of Ca^{2+} transients are critical for the physiological regulation of Ca^{2+}-dependent signaling processes. It was speculated that localized Ca^{2+} responses at the dihydropyridine receptor (DHPR) plays an essential role in fast Ca^{2+} dynamics. Here we report a design of a genetically-encoded Ca^{2+} sensor, CatchER$^+$, that specifically reports rapid local ER Ca^{2+} dynamics with improved Ca^{2+} dynamic range and optical properties. CatchER$^+$ exhibits fast Ca^{2+} on and off rates and Ca^{2+} induced fluorescence increase by elongating life time at the excited state via proton transfer. We have also created an additional sensor CatchER$^+$-JP45 with differential expression close to the ryanodine receptor (RyR1) via the junctional SR protein JP-45 inside the SR lumen via the signal peptide of calreticulin and the ER retention sequence KDEL. Differential drug induced Ca^{2+} releases and ER/SR refilling are observed for various cell types. Furthermore, in electroporated mouse flexor digitorum brevis (FDB) muscle fibers, it's revealed that local Ca^{2+} release via electrical stimulation is significantly greater and faster than that occurring in the SR lumen as assessed using CatchER$^+$ and CatchER$^+$-JP45. Such local Ca^{2+} microdomain transients were removed by knocking out calsequestrin (CaSQ) and/or JP45 revealing its essential role in mediating local fast Ca^{2+} release. These sensors will be invaluable in examining local ER/SR Ca^{2+} dynamics in controlling cellular signaling and excitation contraction coupling in both biological and pathological conditions and facilitate drug development.

Imaging Intraorganellar Ca^{2+} at Subcellular Resolution Using CEPIA

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The endoplasmic reticulum (ER) and mitochondria accumulate Ca^{2+} within their lumens to regulate numerous cell functions. However, determining the dynamics of intraorganellar Ca^{2+} has proven to be difficult. Here, I describe a family of genetically-encoded Ca^{2+} indicators, named calcium-measuring organelle-entrapped protein indicators (CEPIA), which can be utilized for intra-organellar Ca^{2+} imaging. CEPIA, which emit green, red or blue/green fluorescence, are engineered to bind Ca^{2+} at intra-organellar Ca^{2+} concentrations. They can be targeted to different organelles and may be used alongside other fluorescent molecular markers, expanding the range of cell functions that can be simultaneously analyzed. The spatiotemporal resolution of CEPIA makes it possible to resolve Ca^{2+} import into individual mitochondria while simultaneously measuring ER and cytosolic Ca^{2+}. These imaging capabilities were used to reveal differential Ca^{2+} handling in individual mitochondria. CEPIA will provide a useful new approach to further the understanding of organellar Ca^{2+} signals.

Regulation of Astrocyte Calcium Signaling and Gliotransmitter Release by Store-Operated Orai1 Channels

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Store-operated Ca^{2+} release-activated Ca^{2+} (CRAC) channels are a major pathway for calcium signaling in virtually all animal cells and serve a wide variety of functions ranging from gene expression, motility, and secretion to tissue and organ development and the immune response. CRAC channels are activated by the depletion of Ca^{2+} from endoplasmic reticulum (ER) calcium stores, triggered physiologically through stimulation of a diverse set of metabotropic cell-surface receptors. In this talk, I will discuss our recent work on the physiological roles of CRAC channels encoded by Orai1 and STIM1 for the regulation of calcium signaling in hippocampal astrocytes, and CRAC channel-mediated regulation of gliotransmitter release and its ensuing impact on synaptic communication in neighboring neurons. Given the growing recognition of the role of astrocytes for numerous aspects of brain function including synaptic plasticity, cognition, and neurological disease, the results presented here are likely to be relevant for both basic and translational aspects of neuroscience.

The Role of CRAC Channels in Asthma

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Oxford University

Asthma affects three hundred million people worldwide and is the biggest cause of childhood disability in the west. Despite its prevalence, little progress has been made in treating the disease with no new therapy developed over the past twenty years. We have investigated the role of CRAC channels in airway remodeling and immune cell activation that are characteristics of asthma and whether common allergens activate the channels. We find that CRAC channels are activated by a variety of allergens and this leads to prominent downstream signalling, resulting in exocytosis and increased chemokine and cytokine expression. We further show that inhibition of CRAC channels in vivo reduces the severity of allergen-induced asthma in a mouse model. Our results suggest that targeting CRAC channels could be an effective therapy for managing asthma.

Unimolecular STIM-Orai Coupling and Clustering of Orai Channels

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Store-operated Ca^{2+} entry (SOCE) plays a crucial role in receptor-evoked Ca^{2+} signals in almost all cell types. The role of STIM and Orai proteins in mediating SOCE is clear, however, the molecular mechanism of STIM/Orai coupling and the role of other STIM or Orai homologues remains elusive. Recent work from our lab provide new insights on the role of the STIM1 Orai activating region (SOAR) within the STIM1 dimer during binding to Orai1. Using concatenated SOAR dimers containing one or two F394H mutation, we demonstrated that a single functional subunit within a SOAR dimer is necessary for binding to Orai1. We also noticed that concatenated SOAR heterodimers (SH-S) were unable to form Orai1 clusters, whereas cells expressing wild-type dimers retained this ability. We utilized the SOAR domain from the recently discovered splice variant of STIM2 (STIM2.1) to investigate the mechanism of STIM2.1 inhibition on SOCE and its physiological role in receptor-evoked Ca^{2+} signals. Using concatenated heterodimers (SH-S or S2.1-S), we observed profound effects on Orai1 channel clustering, dimer mobility and ICRAC generation when compared to wild-type SOAR1 homodimers (S-S) in cells stably expressing Orai1. The physiological role of clustering Orai1 channels intriguing. Co-expression of full length STIM1 and STIM2.1 profoundly alters the oscillatory response in HEK cells induced by physiological doses of CCh. Importantly, clustering may affect the ability for Orai1 channels to generate local Ca^{2+} microdomains within ER-PM junctions. Our current findings provide strong evidence to support the "unimolecular model" of STIM1-Orai1 coupling. Such coupling allows clustering between Orai channels which appears to have profound significance in the spatial and temporal control of Ca^{2+} signal generation. The STIM2.1 splice variant may be crucial in modulating Ca^{2+} signals through diminished Orai channel clustering.

Huntingtin-Associated Protein 1A Increases SOCE in Medium Spiny Neurons from Transgenic YAC128 Mice, a Model of Huntington's Disease

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Huntington's disease (HD) is a hereditary neurodegenerative disease caused by a polyglutamine expansion within the huntingtin (HTT) gene. It was shown by us and others that mutated huntingtin dysregulated store-operated calcium entry (SOCE), a process in which the depletion of Ca^{2+} from the endoplasmic reticulum (ER) induces Ca^{2+} influx from the extracellular space. We also observed a 3-fold increase in expression of HAP1, a binding partner of HTT, in the striatum of YAC128 mice, a transgenic model of HD.

To identify the mechanism of SOCE dysregulation and possible role of HAP1 we use cultures of medium spiny neurons (MSNs) from YAC128 mice. When expression of HAP1A and HAP1B was silenced by shRNA a decreased activity of SOC channels was detected. In agreement with these observations overexpression of HAP1A in MSNs from YAC128 mice led to enhanced activity of SOC channels after depletion of ER Ca^{2+} by IP_3R1 activation. These observations were confirmed by patch-clamp Ca^{2+} measurements, since HAP1A overexpression in neuronal line SK-N-SH also increased basal activity of SOC channels. Moreover, in YAC128 MSNs overexpressing HAP1A we observed elevated basal Ca^{2+} level, increased activity of IP_3R1, activation of unfolded protein response (UPR), and decrease of the ionomycin-sensitive ER Ca^{2+} pool. The SOCE inhibitor 6-bromo-N-(2-phenylethyl)-2,3,4,9-tetrahydro-1H-carbazol-1-amine hydrochloride, which in our previous studies was identified as a SOCE inhibitor (Czeredys et al., 2016), restored elevated SOCE in YAC128 MSN cultures overexpressing HAP1A. Using immunocytochemistry we now study the possible effect of HAP1A overexpression on co-localization of STIM with Orai proteins in DHPG-induced MSNs from HD and control mice. We suggest that HAP1A is a potential new therapeutic target in HD treatment.

This study was supported by the European Regional Development Fund in the framework of the POMOST project no /2013-8/4 of Foundation for Polish Science for MCz.

Ca^{2+}-Binding Protein ALG-2 May Function in Modulating Ca^{2+} Homeostasis by Interacting with SARAF

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ALG-2 (Apoptosis-Linked Gene 2, gene name: *PDCD6*) is a Ca^{2+}-binding protein containing five serially repeated EF-hand motifs. By interacting with various intracellular proteins in a Ca^{2+}-dependent manner, ALG-2 is involved in apoptosis, cancer development, signal transduction, membrane trafficking, *etc*. However, little is known about ALG-2 functions in the modulation of Ca^{2+} signaling pathways and Ca^{2+} homeostasis. To address these issues, a new luciferase reporter containing nine tandem repeats of an enhancer element of the IL-8 promoter (9 x IL-8) was designed to monitor Ca^{2+}-regulated transcriptional activity of endogenous NFAT (the nuclear factor of activated T cells), and to investigate the effects of ALG-2 on Ca^{2+} signal responses.

Using Nano-Glo Dual-luciferase 9 x IL-8 reporter assay, increased transcriptional activity of NFAT was observed in HEK293 cells stimulated with ionomycin, thapsigargin, or carbachol and ATP. Furthermore, the transcriptional activity of NFAT, which was suppressed by calcineurin inhibitor FK506 and CRAC (calcium release-activated calcium) channel inhibitor BTP2, was much more higher in ALG-2-knockdown HEK293 cells, indicating that ALG-2 may play an important role in modulating Ca^{2+} influx through CRAC channels. Moreover, our obtained results of *in vitro* binding assays including strep-pulldown and co-immunoprecipitation indicated that ALG-2 interacted with SARAF in a Ca^{2+}-dependently manner. SARAF was reported as an ER transmembrane modulator of SOCE (store-operated calcium entry), but no putative Ca^{2+}-binding domain was found in its primary structure. Our findings suggest that ALG-2 is assumed to act as a SARAF-interacting Ca^{2+} sensor in the progress of Ca^{2+}-dependent activation or inactivation of SOCE to regulate Ca^{2+} homeostasis.

Characterization of Stim2b-/- Zebrafish

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STIM proteins are important players in cellular calcium homeostasis as they are required for store-operated calcium entry process (SOCE). STIM2 is highly expressed in rodent brain, however, its properties are far less understood than those of STIM1. One of the reasons is early lethality of *Stim2* knock-out mice. We therefore decided to use zebrafish, which possesses two isoforms: Stim2a and Stim2b. SOCE has not been investigated in the cells of this animal, so we first characterized the expression of the main components of this process. Expression pattern of *stim*s and *orai*s in different tissues of wild-type zebrafish was determined using quantitative RT-PCR. In zebrafish brain we identified the presence of *stim1b* and *stim2a* mRNA (the highest), as well as mRNA of *stim2b* and *stim1a* (the lowest), together with all three isoforms of orais. In the brain and eyes the expression of *stim2b* increased during aging. To investigate the function of Stim2 protein, *stim2b* knock-out zebrafish line was created. These animals do not show any drastic abnormalities in development or fertility. Gene expression profile in wild type and *stim2b-/-* 5dpf zebrafish larvae was explored using RNA-seq analysis. Among hits, several genes related to light sensitivity were found to be differentially expressed in mutants than in wild-types. The results were validated by qRT-PCR analysis. Moreover, behavioral tests showed that *stim2b-/-* larvae react differently than wild-type fish to changes in light intensity. To get a better insight into Stim2 role in the process of light sensitivity, we track calcium responses in zebrafish brain to light stimuli. The stim2b-/- fish were crossed with a transgenic line expressing GCaMP5G calcium probe under the neuronal promoter (HuC). Using Light-Sheet microscopy we conduct *in vivo* calcium imaging in neurons to identify changes as a result of Stim2b absence.

Phenotyping Novel Transgenic Mice Overexpressing ORAI1 in Brain Neurons - FVB/NJ-Tg(Orai1)Ibd

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The most widely accepted risk factor for developing Alzheimer's disease is age. It is suggested that brain ageing is a result of a subtle, but long-lasting dysregulation of calcium ions homeostasis in neurons. Store-operated calcium entry (SOCE) is the major calcium ions influx pathway in non-excitable cells. Recent body of evidence indicates also that SOCE plays an important role in neurons. STIM1 and STIM2 are calcium ions ER sensors mediating the process of SOCE by interacting with the ion channels in the cell membrane - ORAIs. Our group showed that the cytoplasmic resting calcium ions level in primary rat cortical neurons can be modulated by overexpression of STIM proteins. We also detected an enhanced magnitude of calcium ions influx during SOCE in human lymphocytes from SAD patients.

The objective of our research is to understand how elevated basal calcium ions level in neurons contributes to neurodegeneration. We have already obtained transgenic mice with elevated gene expression of STIM1, STIM2 and recently ORAI1 specifically in brain neurons. In one of our papers we described the line overexpressing STIM1 [FVB/NJ-Tg(STIM1)Ibd], which exhibited impairments in synaptic plasticity and behavior. Using neuronal calcium fluorescent imaging combined with electrophysiology and behavioral tests (Open Field, Elevated Plus Maze, Novel-Object Recognition, Fear Conditioning) we now analyze the phenotype of the mouse lines overexpressing ORAI1 [FVB/NJ-Tg(Orai1)Ibd]. Changes in the distribution of AMPA-mediated miniature excitatory postsynaptic currents (mEPSCs) amplitude were observed in organotypic hippocampal slices cultures derived from these mice. Moreover, using local field potential method in acute brain slices derived from six-month old mice we observed alternations in synaptic transmission. This transgenic line might allow us to analyze the involvement of SOCE process on brain dysfunction during ageing.

A Functional Study of MAP Kinases Pathway in Differentiated PC12 Cells Under Impaired Calcium Homeostasis

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Calcium ions are key components initiating a series of vital signal transduction pathways that regulate processes such as cell proliferation, cell differentiation and cell death. A crucial role of plasma membrane Ca^{2+}-ATPase (PMCA) in extruding Ca^{2+} from the cells is well established; however, changes in the expression of particular isoforms are tightly controlled to regulate Ca^{2+} signals. Importantly, the decreasing PMCA activity has been reported in aging neurons. Among 4 isoforms, PMCA2 and PMCA3 display the highest basal activity, and are specific for excitable cells. Using the model of pseudo-neuronal PC12 cells with downregulated PMCA2 or PMCA3 expression we have shown a significant drop in the efficiency of Ca^{2+} removal. We also detected a decreased level of calmodulin, a naturally existing activator of PMCA, on both, mRNA and protein. Our recent data suggested that activation of calcineurin/NFAT pathway may repress CaM genes in PMCAs-downregulated cells. Ca^{2+}/CaM complex regulates many downstream events, including action of MAP kinases, Therefore, in the present study we analyzed the relationship between PMCAs composition and p38, ERK1/2 and JNK activities. Whereas the protein level of the examined kinases appeared to be unaffected in relation to the mock-transfected cells, the activity of p38, ERK1/2, manifested as a phosphorylation index, increased significantly in PMCA-suppressed cells. The results suggest that lowered PMCA ability to expel Ca^{2+} out of cell can modify MAP kinases pathway, which may contribute to adaptive regulation of the cellular response to stress.

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Analysis of CacyBP/SIP and β-Catenin Homeostasis in Cacybp Zebrafish Knockout and YAC128 Mice Model of Huntington's Disease

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We previously detected a 2-fold increase of CacyBP/SIP gene expression encoding calcyclin-binding protein (CacyBP/SIP), and increased level of CacyBP/SIP dimerization in the striatum of YAC128 mice, a model of Huntington's disease (HD), which overexpresses mutant huntingtin (Czeredys et al., 2013). In agreement with the suggested role of CacyBP/SIP in β-catenin degradation we observed a decrease in total protein ubiquitination, and a higher level of β-catenin in the striatum of HD mice as compared to wild-type animals.

Using two different models we now analyze if there is a relationship between the level of CacyBP/SIP dimerization in YAC128 model and β-catenin signaling. One model was the cultures of Medium Spiny Neurons (MSNs) isolated from the striatum of YAC128. In MSNs, in which wild-type CacyBP/SIP was overexpressed using lentiviruses the level of β-catenin and its ubiquitination detected by western blotting was unchanged relative to control vector. To see if only the dimer of CacyBP/SIP can be active in β-catenin homeostasis we analyze MSNs cultures, in which CacyBP/SIP variants with increased or decreased ability to dimerize were overexpressed. To impair or increase the ability of CacyBP/SIP to dimerize, mutations in its coiled-coil dimerization domain were computationally designed using the crystal structure as a template and the Rosetta energy function and molecular dynamics methods. The effect of dimer destabilization (D11A, E14A, and L18A) or stabilization (K21W and a double mutant T30R, S33E) on the level of β-catenin and its ubiquitination are being analyzed.

The second model used is zebrafish. Using CRISPR/Cas9 technology two zebrafish lines with the knockout of cacybp were generated. Preliminary data shows disturbances in β-catenin protein level in total protein extracts from k/o larvae. Both fish lines will be crossed with zebrafish line we generated, which overexpresses GFP-β-catenin fusion protein.

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Involvement of Nitric Oxide-Induced Calcium Release (NICR) Through Type 1 Ryanodine Receptor in Extinction of Cerebellum-Dependent Motor Learning

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Ca^{2+} signals, such as Ca^{2+} influx through voltage-gated Ca^{2+} channels and ionotrophic glutamate receptors, are indicated to be essential for various functions in neuronal systems, including transmitter release and synaptic plasticity. However, functional roles of Ca^{2+} release from intracellular store are not fully understood. We recently identified novel Ca^{2+} release mechanism, nitric oxide-induced calcium release (NICR), in cerebellar Purkinje cells. NICR is mediated by type 1 ryanodine receptor (RyR1), an intracellular calcium release channel, and S-nitrosylation of cysteine at 3636 in RyR1 is essential for the induction of NICR. Although our previous pharmacological studies suggested involvement of NICR in cerebellar synaptic plasticity and neuronal cell death after ischemic brain injury, the physiological function of NICR has yet to be determined. Thus, we generated knock-in mice ($RyR1^{C3636A}$ mice) expressing mutant RyR1 in which cysteine at 3636 was replaced with alanine ($RyR1^{C3636A}$), and examined possible involvement of NICR in cerebellar-dependent function. In the cerebellum of $RyR1^{C3636A}$ mice, NO-induced S-nitrosylation of $RyR1^{C3636A}$ as well as NICR in Purkinje cells were abolished. In addition, a cerebellar synaptic plasticity, long-term potentiation at parallel fiber-Purkinje cell synapse was severely impaired whereas long-term depression in the same synapse seemed to be unaffected. Furthermore, the extinction process, but not acquisition phase, of eyeblink conditioning was impaired in $RyR1^{C3636A}$ mice. These results indicate NICR has essential role in physiological function of nervous systems, including cerebellar-dependent motor learning.

Identification of Ca^{2+} Signaling Components in Proliferating and Differentiating Neurospheres Derived from Primary Neural Stem Cells Isolated from the Zebrafish Brain

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During neurogenesis, undifferentiated neural progenitor cells become differentiated into mature and functional neurons. This process involves: the induction and proliferation of neural progenitors; specification of the progenitors to form committed progenitors; and differentiation into various post-mitotic cell types. Each phase of this process is highly regulated to generate the multiple cell types that eventually populate the mature central nervous system (CNS). The zebrafish is a commonly used model for investigations exploring neurogenesis and CNS regeneration in vertebrates. Furthermore, with the recent development of a reliable protocol for isolating/culturing neural adult stem/progenitor cells from the brain of adult fish, a detailed analysis of the molecular mechanisms underlying neurogenesis is now possible. Using this protocol, we demonstrated that cells dissociated from neurospheres (generated from primary brain cell cultures), can differentiate into cells with neuron, astrocyte or oligodendrocyte characteristics. We have also begun to identify the Ca^{2+} signaling components and investigate the role of Ca^{2+} signaling during proliferation and differentiation of the dissociated neurosphere cells. qPCR was conducted at proliferation day 5, and differentiation days 7 and 14 with primers for specific isoforms of the *ip3r*, *ryr*, *stim*, and *orai* genes and we report that several are upregulated significantly in differentiating cells, when compared with those undergoing proliferation. We also showed via immunocytochemistry that IP_3R (type 1) and RyR (type 2) are expressed in cells with neuronal- or glial-like properties but with different patterns of localization. However, whereas the IP_3R agonist, ATP, induced the generation of Ca^{2+} transients in dissociated cells exhibiting neuron- or glial-like morphology, the RyR agonist, caffeine, did not. Together, our results indicate that although neurospheres comprise a number of distinct cell types, by identifying these cells both from their morphology and via immunolabelling, it is possible to explore the role of Ca^{2+} signaling during differentiation.

Phosphatase and Tensin Homolog (PTEN) Loss is Associated with Changes in Calcium Signalling in MDA-MB-231 Cells

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Phosphatase and Tensin Homolog (PTEN) is the main negative regulator of the phosphoinositide-3-kinase (PI3K)/Akt pathway which is often deregulated in many triple-negative breast cancers, contributing to increased cancer cell proliferation and survival. PTEN loss was recently shown to remodel Ca^{2+} signalling in prostate cancer cells; however this link has not been extensively explored in breast cancer cells. The aim of this project was to assess the effect of PTEN silencing on Akt phosphorylation and Ca^{2+} signalling using MDA-MB-231 cells stably expressing the GCaMP6m genetically-encoded Ca^{2+} sensor (GCaMP6m-MDA-MB-231 cells). GCaMP6m-MDA-MB-231 cells were transfected with non-targeting or PTEN-targeting siRNAs and protein isolated at 48, 72 and 96 h post-transfection. Phosphorylated Akt and total Akt levels were assessed by immunoblotting. For assessment of effect of PTEN silencing on Ca^{2+} signalling, agents which increase Ca^{2+} levels in this cell line (adenosine triphosphate (ATP), trypsin and cyclopiazonic acid (CPA)) were added and relative cytosolic Ca^{2+} changes assessed using a Fluorometric Imaging Plate Reader (FLIPR). We found that PTEN silencing induced Akt phosphorylation at 48, 72 and 96 h post-siRNA transfection. PTEN silencing suppressed IP_3-mediated Ca^{2+} release as a result of ATP and trypsin stimulation, however no significant change in maximum cytosolic free Ca^{2+} induced by CPA was observed. These results are consistent with studies in other cell lines that report PTEN as a regulator of IP_3-induced Ca^{2+} release from the endoplasmic reticulum. Such changes may be through reduced expression, stability or activity of IP_3 receptors. Further studies are required to assess the underlying mechanisms responsible for these Ca^{2+} changes in triple negative breast cancer cells and also the phenotypic consequences of these changes, including responses to apoptotic stimuli.

Investigation of the Role of Ca^{2+} Signaling During Early Embryonic Heart Development in Zebrafish

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In spite of recent medical advancements, heart disease remains a leading cause of death worldwide. While the requirement for Ca^{2+} during normal heart function is well known, more recent evidence suggests that Ca^{2+} might also play a key role in heart morphogenesis. Thus, having a better understanding of the role of Ca^{2+} signaling in normal heart development might help identify novel therapies for congenital and acquired heart diseases, and indicate possible options for pharmacological/genetic intervention to promote heart repair and regeneration. The zebrafish has become a favored vertebrate model for studying cardiac development and disease. This is largely due to several highly successful whole-genome forward-genetic screens that have identified various zebrafish mutants with cardiovascular defects. The introduction of the Ca^{2+} chelator, BAPTA, into 1-cell stage embryos has previously been reported to affect heart development. To investigate this finding in further detail, 1-cell stage *cmlc2*-GFP transgenic embryos (*Tg(cmlc2:GFP)*, which specifically express GFP in cardiac muscle were either injected with BAPTA into the yolk cell, or else incubated with cell-permeable BAPTA-AM. In another series of experiments, embryos were injected with BAPTA into the top of the yolk at 128-cell stage to specifically load it into the yolk syncytial layer. We report that following these various BAPTA treatments, in spite of the gross embryonic morphology being relatively normal, some abnormal heart phenotypes occurred. These included misalignments of the ventricle and atrium, and the complete inversion of the heart chambers. Our results suggest that Ca^{2+} might be important for regulating the organization and migration of cardiac progenitor cells during gastrulation. Thus, we are now investigating where and when Ca^{2+} signaling is most critical for early heart development.

Skeletal Muscle Contractile Force Dysfunction Concurrently with Intracellular Ca^{2+} Dysregulation in a Mouse Model of Type 2 Diabetes

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Type 2 diabetes is characterized by reduced contractile force production and fatigability in skeletal muscle. Maintenance of Ca^{2+} homeostasis during muscle contraction is requisite for optimal contractile function. However, the mechanisms underlying the association between intracellular Ca^{2+} regulation and muscle contractile dysfunction are unclear in type 2 diabetes. Here, we hypothesized that type 2 diabetes induces muscle contractile dysfunction associated with impaired Ca^{2+} regulation in skeletal muscle. Furthermore, we also investigated whether exercise training can improve such dysfunction in skeletal muscle of type 2 diabetes. Skeletal muscle contractile force was assessed by electrical stimulation, and Ca^{2+} dynamics during contraction and pharmacological stimulation were analysed by calcium imaging in C57BLKS/J $LeprCa^{db}/LeprCa^{db}$ mice (db/db; model of human type 2 diabetes). Additionally, the effects of exercise training by treadmill running were investigated. db/db mice displayed decreased tetanic force and fatigue resistance compared with those of healthy controls. Six weeks of exercise training improved muscle contractile dysfunction and fatigability in db/db mice. Furthermore, exercise training improved Ca^{2+} release and accumulation by stimulation in db/db mice. Glucose and insulin tolerance test results were not changed after exercise training in db/db mice. These findings revealed dysfunction of skeletal muscle contractile properties in type 2 diabetes. Furthermore, treadmill exercise training improved such dysfunction in type 2 diabetes via amelioration of intracellular Ca^{2+} dysregulation independent of insulin sensitivity, highlighting the potential to target intracellular Ca^{2+} regulation for type 2 diabetic myopathy treatment.

Complexes of Calcium Waves in Colonic Musculatures of Mice

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In the colon, coordinated motions are required for smooth passage of contents, and unique electrical complexes consisting of rapid and slow oscillations occur. In mechanical aspect, colonic muscle should generate strong propulsive force with tightly regulation of intracellular Ca^{2+}, compared to the small intestine where semi-liquid contents are moderately agitated and slowly transferred.

In this study, we performed Ca^{2+} imaging in transgenic mice of tetO-YC -Nano50::Parvalbumin-tetracycline transactivator, expressing a FRET-based highly sensitive Ca^{2+} indicator in muscle. Musculatures were isolated from a mid part of the colon, and mounted in a sample chamber with the bottom of a heating glass (at 35?C). A pair of emission images of CFP and YFP were simultaneously acquired at ~100 ms intervals using an image splitting optics. Changes in intracellular Ca^{2+} level were estimated by the ratio of CFP and YFP emission images. Degrees of sample movement were estimated by solving the two equations for CFP and YFP emission.

We found that colonic musculatures show Ca^{2+} wave complexes, presumably corresponding to slow and rapid oscillations in the electrical complexes. When a slow increase in baseline Ca^{2+} reached a threshold, Ca^{2+} largely increased for more than ten seconds, overlapping rapid Ca^{2+} transients of ~1-2 Hz. Meanwhile rapid and sustained mechanical movement of musculatures occurred. Application of nifedipine abolished Ca^{2+} wave complexes and mechanical responses, suggesting a major role of L-type Ca^{2+} channels in both generation of spontaneous Ca^{2+}waves and colonic muscle contraction. Additional application of muscarinic agonists partly recovered Ca^{2+} wave complexes, i.e. mainly in rapid Ca^{2+} oscillation component, showing contribution of Ca^{2+} release from the endoplasmic reticulum to rapid Ca^{2+} transients. Thus, we anticipate that the FRET-based imaging techinique is useful to assess the relationship between spatio-temporal Ca^{2+} dynamics and mechanical state of musculatures in variouos regions of the gut.

Visualization of Ca^{2+} Filling Mechanisms Upon Synaptic Inputs in the Endoplasmic Reticulum of Cerebellar Purkinje Cells

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The endoplasmic reticulum (ER) plays crucial roles in intracellular Ca^{2+} signaling, serving as both a source and sink of Ca^{2+}, and regulating a variety of physiological and pathophysiological events in neurons in the brain. However, spatiotemporal Ca^{2+} dynamics within the ER in central neurons remain to be characterized. In this study, we visualized synaptic activity-dependent ER Ca^{2+} dynamics in mouse cerebellar Purkinje cells (PCs) using an ER-targeted genetically encoded Ca^{2+} indicator, G-CEPIA1*er*. We used brief parallel fiber (PF) stimulation to induce a local decrease in the ER luminal Ca^{2+} concentration ($[Ca^{2+}]_{ER}$) in dendrites and spines. In this experimental system, recovery of $[Ca^{2+}]_{ER}$ takes several seconds, and recovery half time depends on the extent of ER Ca^{2+} depletion. By combining imaging analysis and numerical simulation, we show that the intraluminal diffusion of Ca^{2+}, rather than Ca^{2+} reuptake, is the dominant mechanism for the replenishment of the local $[Ca^{2+}]_{ER}$ depletion immediately following the stimulation. In spines, the ER filled almost simultaneously with parent dendrites, suggesting that the ER within the spine neck does not represent a significant barrier to Ca^{2+}diffusion. Furthermore, we found that repetitive climbing fiber (CF) stimulation, which induces cytosolic Ca^{2+} spikes in PCs, cumulatively increased $[Ca^{2+}]_{ER}$. These results indicate that the neuronal ER functions both as an intracellular tunnel to redistribute stored Ca^{2+} within the neurons, and as a leaky integrator of Ca^{2+} spike-inducing synaptic inputs.

Cytosolic Ca^{2+} Dynamics Through the SR is Associated with Pathology of Muscular Dystrophy

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Duchenne (DMD) and the less severe Becker (BMD) muscular dystrophy due to mutations in the *DMD* gene. BMD is a form of dystrophin deficiency that presents with a large spectrum of severities, from borderline DMD to almost asymptomatic cases. A disruption in dystrophin is hypothesized to promote direct Ca^{2+} influx and/or abnormal cytosolic Ca^{2+} ($[Ca^{2+}]_c$) homeostasis, leading to increased volunerability of myofibers to necrosis. $[Ca^{2+}]_c$ regulation is known to be compromised in dystrophic muscle fibers, and several underlying mechanisms have been suggested. These include influx of Ca^{2+} into dystrophic muscle fibers, membrane tears, stretch-activated channels, Ca^{2+} leak channels and leaky Ca^{2+} release channels. Independent clinical reports have revealed that in-frame deletion of exons 45-55 produces an internally shorted, but functional, dystrophin protein and results in a very mild BMD phenotype. However, the regulation including the Ca^{2+} homeostasis in this deletion pattern is not sufficiently clear.

We generated the exons 45-55 deleted dystrophin transgenic/*mdx* (Tg/*mdx*) mice. Muscle function and pathology of Tg/*mdx* mice were restored close to those of wild type mice. On the other hand, the localization of the neuronal type of nitric oxide synthase (nNOS) was changed from the sarcolemma to the cytosol in Tg/*mdx* mice. This altered location led to hyper-nitrosylation of the ryanodine type-1 receptor (RyR1) and similar to *mdx* mice, subsequent increased Ca^{2+} release from the sarcoplasmic reticulum. However, Tg/*mdx* mice displayed restored Ca^{2+} uptake by sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) after activation of RyR1, suggesting that their Ca^{2+} dysregulation can be corrected by SERCA activation. We found that the expression level of sarcolipin, a SERCA-inhibitory peptide, was elevated in *mdx* mice, but normal in Tg/*mdx* mice. Furthermore, knockdown of SLN ameliorated $[Ca^{2+}]_c$ dynamics as well as the dystrophic phenotype in *mdx* mice.

These findings suggest that SLN would be a novel target for DMD therapy.

Ca^{2+} Signals Originate at Immobile IP_3 Receptors Adjacent to the ER-Plasma Membrane Junctions Where Ca^{2+} Entry Occurs

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Inositol 1,4,5-trisphosphate receptors (IP_3Rs) are intracellular Ca^{2+} channels that directly regulate Ca^{2+} release from the ER and thereby also control store-operated Ca^{2+} entry. IP_3Rs open when they bind IP_3 and Ca^{2+}, a property that allows them to propagate Ca^{2+} signals regeneratively. The spatial organization of IP_3Rs determines both this propagation of Ca^{2+} signals between IP_3Rs and the selective regulation of cellular responses. We use gene-editing with TALENs to fluorescently tag endogenous IP_3Rs in mammalian cells, and super-resolution microscopy to determine the geography of IP_3Rs and Ca^{2+} signals within living cells. We show that within ER membranes native IP_3Rs form loose clusters, each typically including about 8 tetrameric IP_3Rs. Most IP_3R clusters are mobile, moved by diffusion and microtubule motors. Although only 31% of IP_3R clusters are immobile, and only a small fraction of these lie close to the plasma membrane, the Ca^{2+} puffs evoked by histamine or photolysis of caged-IP_3 are entirely mediated by these IP_3Rs. Hence, these IP_3Rs are licensed to respond, but they do not readily mix with mobile IP_3Rs. The licensed IP_3Rs reside alongside ER-plasma membrane junctions where STIM1, which regulates store-operated Ca^{2+} entry, accumulates after depletion of Ca^{2+} stores. IP_3Rs tethered close to ER-plasma membrane junctions are licensed to respond and optimally placed to be activated by endogenous IP_3 and to regulate Ca^{2+} entry.

Inhibition of Type I DGK Leads to a Dual Response on Ca^{2+} Signaling in Pancreatic β-Cells

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Diacylglycerol (DAG) is a lipid signal messenger and plays a physiological role in pancreatic β-cells. The intracellular levels of DAG are strictly function. We have previously shown that type I DGK isoforms, DGKα and γ, are highly expressed in β-cells, and that the accumulation of DAG resulting from dysfunction of these DGKs reduces insulin secretion in a PKC-independent manner. We also found that the expression of DGKα and γ is lower in the type 2 diabetes model NSY mice. In the present study, we investigated the effect of DAG accumulation due to type I DGK dysfunction on the regulation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), which is correlated with insulin secretion, in the β-cell line MIN6B. The $[Ca^{2+}]_i$ elevation by glucose stimulation was suppressed by 10 μM R59949, a type I DGK inhibitor, and by 100 μM DiC_8, a membrane permeable DAG analog, in a PKC-independent manner. Moreover, R59949 and DiC_8 reduced voltage-dependent Ca^{2+} channel (VDCC) currents, which were measured by patch clamp technique, suggesting that DAG accumulation due to type I DGK dysfunction suppresses VDCC. In contrast, 1 μM R59949 and 10 μM DiC_8 increased $[Ca^{2+}]_i$ oscillations in a PKC-dependent manner. These results suggest that DAG accumulation due to type I DGK dysfunction has contradictory dual effect on Ca^{2+} signaling in β-cells, depending on the degree of the accumulation; mild accumulation induces a stimulatory effect, whereas excessive accumulation suppresses it.

Molecular Basis for Ca^{2+} and Caffeine Action on the RyR2 Channel and Implications in Disease States

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Type 2 ryanodine receptor (RyR2) is a Ca^{2+} release channel in the sarcoplasmic reticulum of cardiac muscle and plays an important role in excitation-contraction coupling in the heart. Caffeine is a potent activator of the RyR channel which greatly sensitizes the channel to activating Ca^{2+}. However, it remained largely unknown about the mechanism of action of caffeine at molecular level. Recent 3D-structures of RyR1, solved by single-particle cryoEM, have revealed several ligand binding sites including caffeine. These structures should be the key to understanding the mechanism. In fact, mutation in tryptophan residue in the putative caffeine-binding site of human RyR2 (W4645R) is reported to cause catecholaminergic polymorphic ventricular tachycardia (CPVT). Here, we present molecular basis for Ca^{2+}-sensitizing effect of caffeine on RyR2 and implications in disease states. Functional analysis of disease-associated and structure-based artificial RyR2 mutants revealed that hydrophobic interaction between the tryptophan, W4645, in S2S3 domain and isoleucine in C-terminal domain (CTD) to keep CTD apart from core solenoid (CSol) to make Ca^{2+} binding pocket larger and less favorable for Ca^{2+}. Caffeine alters orientation of tryptophan to break the interaction, resulting in movement of CTD toward CSol to make the pocket smaller and more favorable for Ca^{2+} (short-range Ca^{2+} sensitization). Caffeine also increases the affinity for Ca^{2+} of the Ca^{2+}-binding site by conformational changes of CSol via tryptophan-phenylalanine interaction (long-range Ca^{2+} sensitization). We identified two additional CPVT mutations that mimic short-range Ca^{2+} sensitization by different mechanisms. These results suggest that caffeine-binding site is an important regulatory unit in physiological function of RyR2 and that disruption of the regulation will hyper-activate the channel to cause arrhythmogenic diseases.

Transport of Vitamin A Via the STRA6 Receptor is Calcium-Dependent

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Vitamin A is an essential nutrient for all mammals and it is vital for vision. In times of insufficient dietary uptake, retinol delivery to cells occurs primarily via the retinol binding protein receptor, which is encoded by a gene named stimulated by retinoic acid 6 (STRA6). STRA6 is expressed widely with particular abundance in the eye, and mutations in the human gene are linked to Matthew Wood syndrome (MWS), which presents ocular defects ranging from mild microphthalmia to anophthalmia. STRA6 is a 75-kDa multi-pass transmembrane (TM) protein, and a recently determined 3.9-Å resolution cryoEM structure shows it to be a dimer with, unexpectedly, each of its subunits bound to the calcium-binding protein calmodulin (CaM)[1]. CaM binds to the cytoplasmic side of STRA6 in an unconventional arrangement with a helix, termed CaMBP1, of STRA6 found to bind exclusively to the main hydrophobic cleft in the N lobe of CaM. Another helix of STRA6, termed CaMBP0 also binds to the N lobe, on a surface between CaM helices 1 and 4, and interacts additionally with CaMBP1 in a helix-helix crossing mode. The major interaction surface at the CaM-STRA6 interface involves yet another helix of STRA6 termed CaMBP2, which interacts with the hydrophobic groove in the C lobe of CaM much like the canonical 1-5 interaction of the myosin light-chain kinase (MLCK) peptide [2]. With this structure in hand, we measured the effect that peptides derived from STRA6 (CaMP0, CaMP1, and CaMP2) had on the structure, dynamics, and calcium binding to CaM. Overall, these data show that CaMP2 binding to CaM increases the Ca^{2+}-binding affinity of CaM by more than 100-fold and are consistent with a "Binding and Functional Folding" model, which will be discussed. Because CaM was found to have an affinity for Ca^{2+} in the low nanomolar regime when bound to CaMBP2 of STRA6 (CaKD ≈ 100 nM), the calcium-dependence of retinol transport via STRA6 is likely and being explored in more detail inside cells. Support for these studies in funded by the NIH (PI: Mancia; 1R01EY027405).

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Correlation of Molecular Dynamics Analysis and Ca^{2+} Homeostasis in Mutant Type 1 Ryanodine Receptors

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In excitable cells membrane depolarization is translated into intracellular Ca^{2+} signals. Ryanodine receptors (RyRs), located in the sarcoplasmic/endoplasmic reticulum (SR/ER) membrane, are required for intracellular Ca^{2+} release. Malignant hyperthermia (MH) is a disorder of Ca^{2+}-induced Ca^{2+} release (CICR) via the type 1 ryanodine receptor (RyR1) in skeletal muscles. More than 200 mutations have been reported in the RyR1 gene of MH patients. The typical symptoms of MH include a rapid increase in body temperature and induction of a hyper metabolic state with skeletal muscle rigidity. Most of those mutations have been found in three "hot spots" regions of RyR1. However, there were only a few experimental results confirming those mutations being responsible for the increment of the CICR sensitivities. We investigated properties of the RyR1 channels carrying disease-associated mutations at the N-terminal region. HEK293 cells expressing the mutant RyR1 channels exhibited alterations inCa^{2+} homeostasis, i.e., enhanced caffeine sensitivity, decrease of ER Ca^{2+} contents, increases in resting cytoplasmic Ca^{2+} concentration. Molecular dynamics analysis revealed that changes in pattern of electrostatic interaction were correlated with the alteration in Ca^{2+} homeostasis. Increase of electrostatic interaction between D61 and R283 was suggested to play key role to enhance sensitivity to CICR, while, decrease of between D447 and R45 was suggested to leak of Ca^{2+} from the ER. This result suggests that exploration of the functional mutations of RyR1 is effective in preventive diagnosis of patients associated with MH disease.

Molecular Dynamics Study on the Calcium-Dependent Conformational Change of Calmodulin

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Calmodulin is a calcium binding protein that consists of four EF-hand domains. The two EF-lobes of calmodulin, called the N-lobe and the C-lobe, arose from duplication and fusion of a precursor EF-hand. We made a plot of conformational landscape of helix positions of calmodulin lobes by the alignment with the pseudo-two fold axis of the EF-lobe. There are two lines in the plot, each of which is an inferred path of open/close of the EF-lobe based on the observed conformational continuum of EF-lobes from many EF-hand proteins. The inferred path for open/close of the EF-lobe is interpretable in the context of the structural changes of EF-lobe. The plot shows a conformational landscape; this is useful to evaluate the effect of mutation on the conformation of EF-lobe or on the stability and change of EF-lobe by molecular dynamic simulation. The four states of the EF-lobe of calmodulin appeared on two lines in the landscape; these two lines show the trajectory of opening and closing of the EF-lobe. For the N-lobe of calmodulin, the calcium bound form and the apo-forms are on the lower line. The two apo-forms of the C-lobe of calmodulin, with target and without target, are on the upper line. The calcium bound form of the C-lobe is on the lower line.

We analyzed the conformational change induced by calcium or target release from EF-lobe by molecular dynamics. We confirmed that the two lines in our plot are the closing paths of EF-lobes. We found the difference of stability between N and C-lobes in releasing calcium ions; this causes the difference of calcium affinity between two EF-lobes.

The Molecular Mechanism by Which Angiotensin II Activates $Ca_V1.2$ L-type Ca^{2+} Channels in Immature Cardiomyocytes

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Angiotensin II (AngII), the main effector peptide of the renin-angiotensin system, plays important roles in cardiovascular regulation in perinatal mammalians. Here we found that AngII significantly activated $Ca_V1.2$ L-type Ca^{2+} ($Ca_V1.2$) channels in mouse neonatal ventricular cardiomyocytes (NVCM) but not adult ventricular cardiomyocytes (AVCM). This response to AngII was mediated by AT_1 receptors and β-arrestin2. A β-arrestin-biased AT_1 receptor agonist, TRV027, was as effective as AngII in activating $Ca_V1.2$ channels. To elucidate possible involvement of protein kinases in this system, we examined the effects of array of kinase inhibitors in NVCM, revealing that AngII activated $Ca_V1.2$ channels through Src-family tyrosine kinases (SFK) and casein kinase 2 (CK2). In a human embryonic kidney cell line, tsA201 cells, overexpression of CK2$\alpha'\beta$ but not c-Src directly activated recombinant $Ca_V1.2$ channels composed of C-terminally truncated α_{1c}, the distal C-terminus of α_{1c}, β_{2c}, and $\alpha_2\delta_1$ subunits, by phosphorylating threonine 1704 located at the interface between the proximal and the distal C-termini of $Ca_V1.2\alpha_{1c}$ subunits. A cyclin-dependent kinase inhibitor, $p27^{Kip1}$ (p27), inhibited CK2$\alpha'\beta$, and AngII removed this inhibitory effect through phosphorylating tyrosine 88 of p27 via SFK in cardiomyocytes. Coimmunoprecipitation revealed that $Ca_V1.2$ channels, CK2$\alpha'\beta$, and p27 formed a macromolecular complex. These results indicate that stimulation of AT_1 receptors by AngII activates $Ca_V1.2$ channels sequentially through β-arrestin2, SFK, p27, and CK2$\alpha'\beta$ in immature but not adult cardiomyocytes, thereby likely exerting a positive inotropic effect in the immature heart.

The Penta-EF-Hand Protein ALG-2 Functions as an Adaptor in Apoptotic Pathway

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ALG-2 (apoptosis-linked gene 2, also known as PDCD6) is a penta-EF-hand protein and interacts with a variety of proteins in a Ca^{2+}-dependent manner. ALG-2 forms a homodimer and each molecule has at least two binding pockets. Therefore, it is conceivable that the ALG-2 dimer may function as a Ca^{2+}-dependent adaptor. We have recently reported that ALG-2 mediates interactions between ALIX and ESCRT-I (Endosomal sorting complex required for transport-I) in the endocytic pathway and between annexin A11 and Sec31A in the early secretory pathway.

This study has focused on an adaptor function(s) of ALG-2 in the apoptotic pathway since we have recently identified CDIP1 (cell death-inducing p53 target protein 1) as an interacting protein for ALG-2. CDIP1 is a p53 target protein with proapoptotic activities. It contains a Pro-rich region, including a type 2 ALG-2-binding motif (ABM-2), and a binding motif for the ESCRT-I component TSG101. We first performed immunoprecipitation experiments using HEK293 cells. Endogenous ALG-2 was co-precipitated with SGFP2-fused CDIP1 (GFP-CDIP1) in the presence of Ca^{2+}, but not in the presence of EGTA. The interaction was largely prevented by mutations in ABM-2. Overexpression of GFP-CDIP1 in HeLa cells induced cleavage of PARP, indicating activation of caspase 3/7 in the cells. The deletion of ABM-2 resulted in a slight reduction in the cleavage of PARP. In contrast to ALG-2, TSG101 could be co-precipitated with GFP-CDIP1 in the presence of EGTA, but addition of Ca^{2+} increased significantly the amount of TSG101 in the immunoprecipitates. We also found no or little interactions between GFP-CDIP1 and previously identified ALG-2-interacting proteins, including ALIX, annexin A11 and Sec31A. Therefore, ALG-2 may specifically help bridge CDIP1 and TSG101 in a Ca^{2+}-dependent manner. This adaptor function of ALG-2 expands our knowledge as to the CDIP1-mediated proapoptotic pathway.

Effects of Altered PMCA Composition on IP_3 Receptors Expression Level and CCL5 - Induced Response in Differentiated PC12 Cells

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Impaired calcium homeostasis contributes to development of neurodegeneration induced by pro-inflammatory chemokines. These recognized mediators, including chemokine CCL5, operate by specific membrane receptors coupled to G-protein. One of the mechanisms involves calcium mobilization from intracellular stores due to activation of phospholipase C and subsequent generation of IP_3. A set of intracellular receptors - directly IP_3R and indirectly RyR, transmits chemokine signals by releasing of sequestered Ca^{2+} into cytosol. Crucial role in termination of Ca^{2+} signal is played by plasma membrane Ca^{2+}-ATPase (PMCA), the most sensitive calcium detector. The enzyme exists in four isoforms with diverse tissue distribution. PMCA1 and PMCA4 are ubiquitously represented, whereas PMCA2 and PMCA3 appear to be neuron-specific. Using stable-transfected differentiated PC12 cells with downregulated PMCA2 or PMCA3 isoforms we assayed whether modified profile of calcium pumps can influence cell response on CCL5 treatment. The presence of potentially CCL5-sensitive receptors (CCR1, CCR3, CCR5) was verified by Western blot analyses. The experiments in Ca^{2+}-free buffer with specific inhibitors - U73122 for PLC and 2APB for IP_3R - confirmed that all lines responded to CCL5 by PLC/IP_3 pathway. However, there were differences in CCL5-induced calcium transients generation, and half-time required for Ca^{2+} clearance was altered. Further analysis showed that suppression of PMCAs altered the expression level of IP_3 receptors in isoform-specific manner by yet unidentified regulatory cascade. It suggests that composition of PMCA may be an important factor in regulation of calcium-dependent expression IP_3 receptors, thereby may affect the cell response to signaling induced by chemokines.

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Arrhythmogenic Calmodulin Mutations Differentially Impair Inhibition of RyR2-mediated Ca^{2+} Release

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Calmodulin (CaM) is a ubiquitously expressed Ca^{2+} binding protein that transduces changes in cytosolic Ca^{2+} concentrations into allosteric regulation of target proteins. CaM regulates many proteins that control excitation-contraction coupling and intracellular Ca^{2+}-cycling in cardiomyocytes. Consequently, CaM mutations perturb their CaM- and Ca^{2+}-dependent regulation and cause life threatening arrhythmias, including CPVT and LQTS. To date, 15 arrhythmogenic CaM mutations have been identified, but their disease mechanisms are largely undefined. One proposed mechanism involves the impairment of CaM-dependent inhibition of intracellular Ca^{2+} release through the ryanodine receptor type 2 (RyR2). However, whether arrhythmogenic CaM mutations generally impair RyR2 function is unclear. Here we investigated the effect of the 15 arrhythmogenic CaM mutations on the CaM-dependent RyR2 inhibition. We found that all the mutations, except CaM-F142L, partially reduced the threshold for termination of store-overload induced Ca^{2+} release (SOICR) in RyR2 expressing HEK293 cells, and 7 mutations also reduced the threshold for SOICR activation. We also found, that the CaM mutations, opposite wild type CaM, did not inhibit RyR2 in permeabilized cells during sustained 1 micromolar Ca^{2+} stimulation, and 8 CaM mutations even promoted Ca^{2+} release. Biophysical experiments showed that reduced CaM binding to Ca^{2+} or to the RyR2 CaM-binding domain does not fully correlate with impaired RyR2 inhibition. Our results demonstrate that arrhythmogenic CaM mutations differentially impair RyR2 function, and that insufficient RyR2 inhibition is likely an underlying component of CaM-linked arrhythmias, including CPVT, LQTS or IVF.

TPC2-Mediated Ca^{2+} Signaling is Required for the Establishment of Synchronized Connectivity Within the Zebrafish Embryonic Spinal Circuitry

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In zebrafish, one of the first observed behavioral activities is the spontaneous coiling of the trunk. This begins at ~17 hours post-fertilization (hpf) and coincides with the spontaneous activity of the primary neurons in the spinal cord as well as Ca^{2+} transients generated in the slow muscle cells in the trunk. It has previously been reported that in the primary motor neurons (PMNs), initial spontaneous stochastic Ca^{2+} transients are associated with action potentials, which during early spinal circuit development, transform from being slow and uncorrelated, to being rapid, synchronized, and patterned by ~24 hpf. However, it remains unclear as to how the PMN Ca^{2+} signals are generated and how they might in turn regulate the maturation of the spinal network. Nicotinic acid adenine dinucleotide phosphate (NAADP) is a potent Ca^{2+} mobilizing messenger that targets two-pore channels (TPCs) to mediate Ca^{2+} release from endolysosomal compartments. In this study, we demonstrated that TPC type 2 (TPC2)-mediated Ca^{2+}-release is required for the establishment of highly synchronized connectivity within the zebrafish embryonic spinal circuitry. Using the SAIGFF213A;UAS:GCaMP7a double-transgenic line of fish (a kind gift from Prof. Koichi Kawakami), which expresses GCaMP7a in the caudal primary motor neurons (CaPs), Ca^{2+} transients were visualized starting from the early stages of spontaneous activity at ~18 hpf. We report that TPC2 inhibition resulted in a decrease in the frequency and amplitude, as well as the ipsilateral and contralateral correlation, of the CaP Ca^{2+} transients, indicating a significant disruption of the maturing spinal circuitry. Furthermore, inhibition of the neural action potentials via treatment with MS-222 resulted in decreased NAADP production. Together, our new data suggest a novel function for NAADP/TPC2-mediated Ca^{2+} signalling in the development, coordination, and maturation of the spinal network in zebrafish embryos.

Physical Interaction of Junctophilins and the C-terminus of Ca_V1.1 Subunits is Crucial for the Excitation-Contraction Coupling of the Skeletal Muscle

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In skeletal myocytes, Ca_V1.1 L-type calcium channels (LTCC) form a complex with ryanodine receptors at the junctional membrane (JM) where the sarcolemma is closely apposed to sarcoplasmic reticulum (SR) membranes. Junctophilins (JPs) are a molecule known to stabilize the JM complex by bridging the sarcolemma and SR. However, it is uncertain how JPs determine this localization of LTCC and support the excitation-contraction coupling of the skeletal muscle. We first knockdowned JPs in cultured myotubes. Suppression of JPs disturbed the proper JM-targeting of LTCC and a robust calcium transient in response to electrical stimulation. Co-immunoprecipitation and GST pull down assays demonstrated that JPs physically interacted with a part of the C-terminus of Ca_V1.1 subunits of LTCC. Transient expression of a JP1 mutant lacking its transmembrane domain (JP1ΔTM) inhibited the proper JM-targeting of Ca_V1.1 in cultured myotubes, indicating a dominant negative effect of the mutant. To examine the in vivo effect of JP1, JP1ΔTM was transduced in the tibialis anterior (TA) or flexor digitorum profundus (FDB) muscle of living mice by using an adeno-associated virus-mediated gene delivery system. Ten days after the infection, the specific force of the TA muscle was dramatically reduced compared to the control. Moreover, Ca^{2+} imaging assay in the FDB fibers showed that JP1ΔTM significant decreased the peak amplitude of calcium transients elicited by electrical stimulations without reducing the SR Ca^{2+} content. Consistent with these results, the proximity ligation assay revealed that the physical coupling between LTCC and RyR in the FDB fibers were significantly decreased by JP1ΔTM. From these results, we conclude that the physical interaction between JPs and the C-terminus of Ca_V is crucial for the excitation-contraction coupling of the skeletal muscle.

The Calcium-Binding Protein ALG-2 Binds to Novel ALG-2-Interacting Proteins, MISSL and MAP1B, and Regulates the Secretory Pathway

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The calcium-binding protein ALG-2 is a 22-kDa protein possessing the penta-EF-hand (PEF) domain. ALG-2 has been reported to be involved in the wide range of cellular processes, such as secretion, apoptosis, and vesicular trafficking. Previous studies have suggested the potential involvement of calcium and of ALG-2 for regulation of the early secretory pathway, *i. e.*, endoplasmic reticulum (ER)-to-Golgi transport, although molecular mechanism for this regulation is poorly characterized yet.

We have previously searched ALG-2-interacting protein candidates through *in silico* screening based on ALG-2 recognition motifs. We revealed MISSL (microtubule-interacting and spindle-stabilizing-like), a previously uncharacterized protein, as a novel interacting protein of ALG-2. Consistent with this, GFP-tagged MISSL was recruited to ER exit sites (ERES) by ALG-2 upon intracellular calcium rise. Knockdown of MISSL affected distributions of components of ERES, the ER-Golgi intermediate compartment (ERGIC), and Golgi. We also found that knockdown of either ALG-2 or MISSL resulted in reduction of secretion of SEAP (secreted alkaline phosphatase), a model secretory protein. Simultaneous knockdown of ALG-2 and MISSL did not show any additive effect on reduction of secretion of SEAP compared with that of respective single knockdown. Together, these results suggest that ALG-2 and MISSL play a role in the same pathway regulating the secretion. Furthermore, we identified MAP1B (microtubule-associated protein 1B) as a binding protein for ALG-2 and MISSL, and demonstrated the calcium-dependent ternary complex formation. Interestingly, knockdown of MAP1B recovered SEAP secretion that was reduced in the knockdown of ALG-2 or MISSL.

Thus, these results suggest that calcium-dependent formation of a ternary complex composed of ALG-2, MISSL and MAP1B acts as a regulatory mechanism for the calcium-dependent secretion process.

Regulation of Spinogenesis in Mature Purkinje Cells via mGluR/PKC-Mediated Phosphorylation of CaMKII β

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The cerebellar cortex and its sole output, Purkinje cell, is essential for motor coordination and learning. Dendritic spines of Purkinje cells form excitatory synapses with parallel fiber terminals, which are the primary sites for cerebellar synaptic plasticity. Nevertheless, how density and morphology of these spines are properly maintained in mature Purkinje cells is not well understood. Here we show an activity-dependent mechanism that represses excessive spine development in mature Purkinje cells. We found that CaMKII β promotes spine formation and elongation in Purkinje cells through its F-actin bundling activity. Importantly, activation of group I mGluR, but not AMPAR, triggers PKC-mediated phosphorylation of CaMKII β, which results in dissociation of the CaMKII β /F-actin complex. Defective function of the PKC-mediated CaMKII β phosphorylation promotes excess F-actin bundling and leads to abnormally numerous and elongated spines in mature IP_3R1-deficient Purkinje cells. Thus, our data suggest that phosphorylation of CaMKII β through mGluR/IP_3R1/PKC signaling pathway represses excessive spine formation and elongation in mature Purkinje cells.

Hypoxic Stress Facilitates Cell Proliferation via Dynamin2-Kir2.1 Pathway in Brain Capillary Endothelial Cells

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The blood-brain barrier (BBB) is mainly composed of brain capillary endothelial cells (BCECs), astrocytes and pericytes. The enhancement of BCEC proliferation by brain ischemia may be partly responsible for the ischemia-induced damage of BBB. Ca^{2+} signaling, particularly Ca^{2+} influx, is a key event of this enhancement in BCECs. However, the underlying mechanisms for Ca^{2+} influx are not clear yet. In the present study, t-BBEC117 cells, an immortalized bovine brain endothelial cell line, were cultured under hypoxic conditions at 4~5% oxygen for 72 h. This hypoxic stress caused membrane hyperpolarization by up-regulating functional expression of Kir2.1 that forms Ba^{2+}-sensitive inward rectifier K^+ current in t-BBEC117 cells. The expression of Kir2.1 was significantly up-regulated at protein level but not at mRNA level after the hypoxic culture. We hypothesized the involvement of dynamin2 that controls surface expression of membrane proteins. Hypoxic culture up-regulated dynamin2 expression in t-BBEC117 cells. Experiments using a dynamin inhibitor or dominant-negative form of dynamin2 revealed that the up-regulation of dynamin2 under hypoxia increased the enhancement of both Kir2.1 membrane expression and Ba^{2+}-sensitive currents in t-BBEC117 cells. Ca^{2+} imaging analysis revealed that the hypoxic stress enhanced store-operated Ca^{2+} (SOC) entry, which was significantly reduced by Kir2.1 inhibition. On the other hand, the expression of SOC channels such as Orai1, Orai2, and transient receptor potential (TRP) channels was not affected by the hypoxic stress. MTT assay showed that the Kir2.1 inhibition significantly attenuated hypoxia-induced cell proliferation in t-BBEC117 cells. Our results demonstrated that the hypoxic stress increases dynamin2 expression and facilitates Kir2.1 surface expression, resulting in hyperpolarization of membrane potential in BCECs. This hyperpolarization increases Ca^{2+} influx and facilitates BCEC proliferation and may lead to result in BBB disruption (Yamamura et al., BBRC, 2016).

A New Splice Variant of Large-Conductance Ca^{2+}-Activated K^+ (BK) Channel α Subunit Alters Human Chondrocyte Function

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Changes in intracellular calcium concentration ($[Ca^{2+}]_i$) modulate chondrogenesis, proliferation, cell death and baseline anabolic and catabolic activities in chondrocytes. Under pathophysiological conditions, like osteoarthritis (OA), histamine release from resident mast cells enhances the production of pro-inflammatory mediators and the activities of matrix degrading enzymes. Specific patterns of $[Ca^{2+}]_i$ changes are considered as essential components initiating and/or progressing the disease. In an established model cell-line of the human chondrocyte, OUMS-27 cells, large-conductance Ca^{2+}-activated K^+ (BK) channels are involved in the enhancement of histamine-induced Ca^{2+} influx by the activation of Ca^{2+} release-activated Ca^{2+} (CRAC) channels consisting of Orai1-Orai2 heteromers and STIM1 via the modulation of membrane potential.We have identified a novel BKα splice variant (BKαΔe2) in OUMS-27 cells. Although BKαΔe2 lacks exon2 that codes the intracellular S0-S1 linker (Glu127-Leu180), significant expression was detected in several human tissues including chondrocytes of OA patients and in mice tissues as well. BKαΔe2 channels are not expressed on plasma membrane (PM), but can traffic to PM after forming hetero-tetramer units with wild-type BKα (BKαWT). These hetero-tetramers of BKα have a smaller single channel conductance and exhibit lower trafficking efficiency than BKαWT homo-tetramers in a stoichiometry-dependent manner. BKαΔe2 knockdown in OUMS-27 chondrocytes increased BK current density, and augmented the responsiveness to histamine assayed as cyclooxygenase-2 gene expression. These findings provide significant new evidence that BKαΔe2 can modulate cellular responses to physiological stimuli in human chondrocyte and contribute under pathophysiological conditions, such as OA.

TPC2-Mediated Ca^{2+} Signaling is Required for the Development of Slow Muscle Cells in Zebrafish Embryos

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We recently demonstrated a critical role for two-pore channel type2 (TPC2)-mediated Ca^{2+} release during the differentiation of slow (skeletal) muscle cells (SMC) in intact zebrafish embryos, via the introduction of a translational-blocking morpholino antisense oligonucleotide (MO). Here, we extend our study and demonstrate that knockdown of TPC2 with a non-overlapping splice-blocking MO, knockout of TPC2 (via the generation of a *tpcn2*^{dhkz1a} mutant line of zebrafish using CRISPR/Cas9 gene-editing), or the pharmacological inhibition of TPC2 action with bafilomycin A1 or *trans*-ned-19, also lead to a significant attenuation of SMC differentiation, characterized by a disruption of SMC myofibrillogenesis and gross morphological changes in the trunk musculature. When the morphants were injected with *tpcn2*-mRNA or were treated with IP_3/BM or caffeine (agonists of the inositol 1,4,5-trisphosphate receptor (IP_3R) and ryanodine receptor (RyR), respectively), many aspects of myofibrillogenesis and myotomal patterning (and in the case of the pharmacological treatments, the Ca^{2+} signals generated in the SMCs), were rescued. STED super-resolution microscopy revealed a close physical relationship between clusters of RyR in the terminal cisternae of the SR, and TPC2 in lysosomes, with a mean estimated separation of ~52-87 nm. Our data, therefore, add to the increasing body of evidence, which indicate that localized Ca^{2+} release via TPC2 might trigger the generation of more global Ca^{2+} release from the sarcoplasmic reticulum via Ca^{2+}-induced Ca^{2+} release.

C-Terminal Splice Variants of P/Q-type Ca^{2+}Channel CaV2.1 α1 Subunits are Differentially Regulated by Rab3-Interacting Molecule Proteins

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Rab3-interacting molecules (RIMs) are known as scaffold proteins involved in neurotransmitter release by voltage-dependent Ca^{2+} channels (VDCCs). RIMs confer voltage-dependent inactivation (VDI) suppression activity and anchoring of synaptic vesicles to the VDCCs. Here, we report on interaction and electrophysiological effect of RIMs and exons 44 and 47 of C-terminal of P/Q-type VDCC α1 subunit (Cav2.1), which also known as a presynaptic proteins-interacting region. Co-immunoprecipitation experiments have demonstrated association of RIM1α and RIM2α to Cav2.1 exons 40-47, however, this interaction was abolished due to alternative splicing of protein regions encoded by the exons. VDI suppression was also found to be weakened by RIM2α to a level comparable to that of RIM1α-mediated VDI suppression, which was unaffected in the exons-deleted Cav2.1 variant. Studies from exon 47-deleted mutant have identified involvement of 17 amino acid residues at the C-terminal side of a polyglutamine stretch as being critical for the potentiated VDI-suppression characteristic of RIM2α. It has further suggested that Cav2.1 proteins are able to distinguish α-RIM isoforms in VDI suppression of P/Q-type VDCC currents, as resulted from Cav2.1 C-terminal domain-RIMs interactions.

Mutual Antagonism Between IP_3 and Anti-Apoptotic Bcl-2 Modulates IP_3R Activity by Competing for the Ligand-Binding Domain

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Bcl-2 protein has emerged as critical regulator of intracellular Ca^{2+} dynamics by directly targeting and inhibiting the IP_3 receptors (IP_3Rs), intracellular Ca^{2+}-release channels. Here, we show that inhibition of IP_3R activity by Bcl-2 was counteracted by high concentrations of IP_3 or agonist in permeabilized cells and in single-channel recordings or in intact cells, respectively. This could be attributed to the binding of Bcl-2's BH4 domain to the ligand-binding domain (LBD) of the IP_3R, shown experimentally and fitting in silico models. In silico analysis revealed steric hindrance by IP_3 of the Bcl-2 binding to LBD. Consistent with this, the binding of Bcl-2 and its BH4 domain to the LBD was antagonized by IP_3 or adenophostin A (AdA). Vice versa, Bcl-2 or its BH4 domain interfered with IP_3 binding to the IP_3R or to the LBD, respectively. Thus, by competing for the LBD, IP_3 and Bcl-2 mutually affect their impact on IP_3R function: high concentrations of IP_3 prevents IP_3R inhibition by Bcl-2 and Bcl-2 prevents IP_3 binding to the IP_3R. This study provides a novel mechanism for IP_3R inhibition by Bcl-2 and reveals that the IP_3R-inhibitory properties of Bcl-2 are counteracted by high concentration of IP_3 or strong agonist stimulation

Secretion of the Phosphorylated Form of S100A9 From Neutrophils is Essential for the Pro-Inflammatory Functions of Extracellular S100A8/A9

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S100A8 and S100A9 are members of the S100 family of cytoplasmic EF-hand Ca^{2+}-binding proteins and are abundantly expressed in the cytosol of neutrophils. Mostly found under heterodimeric form, S100A8/A9 have various intracellular roles but can also be secreted in the extracellular environment where they act as alarmins amplifying the host inflammatory response. While the intracellular activity of S100A8/A9 was shown to be regulated by the phosphorylated form of S100A9, the importance of this post-translational modification on the extracellular activity of S100A8/A9 has not yet been extensively studied. Therefore, we focused our work on the role of phosphorylation of secreted S100A9 and its impact on the pro-inflammatory function of neutrophils.

First, we characterized the secretion of S100A8/A9 under different stimulatory conditions and investigated the phosphorylation state of secreted S100A9. Our results on neutrophil-like differentiated HL-60 cells (dHL-60) and purified human neutrophils show a time-dependent secretion of S100A8/A9 with a phosphorylated-state of S100A9 when induced by PMA. Moreover, our data suggest that S100A8/A9 are secreted via the process of neutrophil extracellular traps (NETs). Next, we investigated the impact of S100A9 phosphorylation on pro-inflammatory cytokine expression and secretion in dHL-60 cells. For that, time course experiments with S100A8/A9 or S100A8/PhosphoA9 were performed and the expression and secretion levels of IL1a, IL1b, IL6, TNFa, CCL2, CCL3, CCL4 and CXCL8 were measured by real-time PCR and cytometry bead array, respectively. Our results demonstrate that only the phosphorylated form of the S100A8/A9 complex induces pro-inflammatory cytokine expression and secretion. Finally, we were able to show that S100A8/PhosphoA9 is inducing cytokine secretion through TLR4 signaling.

These results provide a new insight into the neutrophil pro-inflammatory response to S100A8/A9 and their possible involvement in the pathogenesis of inflammatory diseases.

Identification and Characterization of a Centrosomal Protein, FOR20 as a Novel S100A6 Target

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S100A6 (also known as calcyclin), a member of the S100 family of EF-hand calcium-binding proteins, was first discovered as the growth-regulated gene, 2A9. The physiological functions of S100A6 were thought to be mediated by interactions with its target proteins including Annexin II, VI, XI, CacyBP/SIP, protein phosphatase 5 [1] and the receptor for advanced glycation end products (RAGE). The growing number of S100A6 target proteins may indicate the increasing number of physiological functions ascribed to S100A6 and the existence of novel Ca^{2+}/S100A6-dependent signaling pathways. Recently, we developed a novel genome-wide screening method for the identification of calmodulin-binding targets using the Protein Active Array® (Cell Free Sciences Co., Ltd, Ehime, Japan) carrying recombinant human proteins [2]. In this report, we screened 19,676 human full-length cDNA-derived GST-fusion proteins using a biotinylated S100A6-binding assay and discovered a centrosomal protein, FOR20 (FOP-related protein of 20 kDa) as a novel S100A6-binding protein [3]. Binding experiments revealed that S100A6 interacts with the N-terminal region (residues 1-30) of FOR20 in a Ca^{2+}-dependent manner *in vitro* and in living cells. Several S100 proteins including S100A1, A2, A4, A11, B also exhibited Ca^{2+}-dependent interactions with FOR20 as well as S100A6. We found that two distantly related centrosomal proteins, FOP and OFD1, also possess N-terminal regions with a significant sequence similarity to the putative S100A6-binding site (residues 1-30) in FOR20 and are capable of binding to S100A6 in a Ca^{2+}-dependent manner. Taken together, these results may indicate that S100A6 interacts with FOR20 and related centrosomal proteins through a conserved N-terminal domain, suggesting a novel Ca^{2+}-dependent regulation of centrosomal function.

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Characterization of Protein Domains Important for the Flubendiamide Action in the Lepidopterous Ryanodine Receptors

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Diamide insecticides, such as flubendiamide, show selective activity against lepidopterous insects, the most widely distributed major pest of crucifers. This interesting selectivity of diamide insecticides is underlain by their exclusive action on ryanodine receptor (RyR) channels, causing sustained cytoplasmic Ca^{2+} elevation, leading to rigid muscle and feeding cessation. However, the molecular mechanisms underlying the species-specific action of diamide insecticides are still elusive. Here we studied how lepidopterous RyRs are selectively recognized and regulated by flubendiamide using silkworm RyR (sRyR). In sRyR, replacement of the divergent region 1 (DR1), poorly conserved among species, with that from rabbit RyR2 significantly impaired the Ca^{2+} release response to flubendiamide, but spared receptor sensitivity to caffeine, a universal RyR activator. Nano LC-MS/MS analysis after flubendiamide-based affinity labeling probe, flubendiamide-AI, labeling revealed that the labeling site itself was localized in the central domain adjacent to DR1 in the cytoplasmic region of sRyR. Importantly, mutation of the labeling site retained an intact response of the receptor to flubendiamide but abolished incorporation of flubendiamide-AI in sRyR. Furthermore, the mutation G4866E, corresponding to the diamide-resistant mutation identified at the C-terminal end of the S4 transmembrane segment in RyR of the diamondback moth, abolished the flubendiamide response but did not disrupt incorporation of flubendiamide-AI labeling or the caffeine response in sRyR. Our findings suggest that flubendiamide directly binds to DR1 with its aliphatic amide moiety oriented toward T3906 of the central domain, which serves as a reaction site for the acyl imidazole moiety of flubendiamide-AI in sRyR. Structural changes induced by this interaction are likely to be transmitted to the S4 segment via G4866 to open the Ca^{2+}-permeable channel pore of sRyR.

Configuration of SNARE Proteins and Calcium-Dependent Exocytosis

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Intracellular calcium concentration controls hormone secretion and neurotransmitter release. We have investigated the SNARE assembly at the presynaptic terminals of cortical neurons and plasma membrane of pancreatic beta cells, using two-photon fluorescence lifetime imaging (2p-FLIM) of Forster resonance energy transfer (FRET). SNAP25 is one of the SNARE proteins at the target plasma membrane, and it carries two α helixes, SN1 and SN2. We measured intermolecular FRET ratio between mtq-SNAP25 and SN1-Venus-SN2 in the axonal boutons. High binding fraction (BF:13%) was observed, colocalized with a molecular marker of active zone, Bassoon. In contrast, in the pancreatic islet preparations, no significant assembly was detected. Such data indicated the domain-swapped model; where SN1 bound to the SN2 of the other SNAP25 molecule, and formed oligomer. We further utilized the dissociated culture of neurons from SNAP25 knock-out mice. We rescued the synaptic transmission by viral transfection of FRET probes, and prepared the situation where fluorescent SNAP25 selectively functioned. The synaptic transmission was measured with inhibitory postsynaptic current (IPSC) triggered with electrical field stimulation. The C-terminal deletion mutant revealed reduced binding fraction, and IPSC was triggered with longer delay. The longer linker between two α helixes showed similar binding fraction as in control cases, but the kinetics of transmission was delayed. The experiment with caged-calcium stimulation also revealed that longer linker mutations (>10 amino acids) markedly inhibited synaptic transmission. Our data suggested that the domain swapping of SNAP25 with the linker of appropriate length was necessary for proper tension around the fusion pore, and for triggering ultrafast exocytosis in presynaptic terminals.fluorescence lifetime imaging (2p-FLIM) of Forster resonance energy transfer (FRET). SNAP25 is one of the SNARE proteins at the target plasma membrane, and it carries two α helixes, SN1 and SN2. We measured intermolecular FRET ratio between mtq-SNAP25 and SN1-Venus-SN2 in the axonal boutons. High binding fraction (BF:13%) was observed, colocalized with a molecular marker of active zone, Bassoon. In contrast, in the pancreatic islet preparations, no significant assembly was detected. Such data indicated the domain-swapped model; where SN1 bound to the SN2 of the other SNAP25 molecule, and formed oligomer. We further utilized the dissociated culture of neurons from SNAP25 knock-out mice. We rescued the synaptic transmission by viral transfection of FRET probes, and prepared the situation where fluorescent SNAP25 selectively functioned. The synaptic transmission was measured with inhibitory postsynaptic current (IPSC) triggered with electrical field stimulation. The C-terminal deletion mutant revealed reduced binding fraction, and IPSC was triggered with longer delay. The longer linker between two α helixes showed similar binding fraction as in control cases, but the kinetics of transmission was delayed. The experiment with caged-calcium stimulation also revealed that longer linker mutations (>10 amino acids) markedly inhibited synaptic transmission. Our data suggested that the domain swapping of SNAP25 with the linker of appropriate length was necessary for proper tension around the fusion pore, and for triggering ultrafast exocytosis in presynaptic terminals.

Deregulation of Calcium Feedback via Calcium Sensor Proteins, GCAPs, Triggers Photoreceptor Death in Dominant Cone-Rod Dystrophy (CORD6) Mouse Model

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Calcium sensor EF-hand proteins GCAPs regulate photoresponse in retinal rods and cones via negative calcium feedback, by activating retinal membrane guanylyl cyclase (RetGC) in the light, when Ca^{2+} concentrations fall, and decelerating the cyclase in the dark, when Ca^{2+} concentrations rise. Substitutions of Arg838 in human RetGC1 linked to a severe congenital blindness, autosomal dominant cone-rod dystrophy type 6 (CORD6), stabilize the cyclase complex with the 'activator' *versus* 'inhibitor' state of GCAP and thus reduce Ca^{2+} sensitivity of the cyclase regulation by GCAPs *in vitro*. The R838S RetGC1 transgenically expressed in mouse rod outer segments *in vivo* remains activated by GCAPs even at calcium concentrations typical for dark-adapted rods and thus elevates influx of Na^{+} and Ca^{2+} through the cGMP-gated channels of the plasma membrane in the dark. Sensitivity and the shape of photoresponses recorded from individual rods change consistently with dysregulation of the negative calcium feedback on R838S RetGC1. Rod vision in R838S mouse retinas rapidly declines between 1 and 6 months of age due to severe degeneration of the diseased rods. In contrast, responses from cones, not targeted for the R838S RetGC1 expression and serving as a control group of photoreceptors in the same retinas, remain strong even at the time when rod responses become suppressed. Elimination of the Ca^{2+} feedback on the cyclase, by breeding the R838S RetGC1 mice into GCAPs knockout background, rescues the diseased rods from degeneration. Our study presents a new experimental model for exploring cellular mechanisms of the CORD6 blindness. It shows that CORD6 is a 'phototransduction disease', triggered by abnormality in the negative Ca^{2+} feedback on guanylyl cyclase mediated by GCAPs.

Designing and Validation of Calcium Binding Site in EF-Hand Motif and Comparative Insights into the Site-Specific Binding Affinity

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Different Ca^{2+}-binding proteins display different levels of affinities for calcium. Many functions of these proteins, in general, depend on their affinity for Ca^{2+}. Earlier, we devised a method to identify and classify the EF-hand loops which are present as highly conserved motifs and are very important class of proteins families. Based on the input sequences, we predicting the loop structure in EF-hand motif and classified the loop into different levels of affinities as high, low or none. The classifier showed high accuracy for the data available from the literature.

Here, we applied two new scoring schemes to 1) refine the predictions and 2) understand the role of individual residues in EF-loop in calcium binding. In order to validate the predictions, we designed a unique EF-hand loop capable of binding calcium with high affinities by mutating residues on the basis of high SVMMar and PSMLogL scores. The unique sequence was incorporated in the Entamoeba histolytica Calcium binding protein1 (EhCaBP1) EF-hand loop 2 using site directed mutagenesis. The sixty-six amino acid residues long protein containing modified second EF-hand loop and the EhCaBP1-Wt with low affinity loops were studied for calcium binding properties using ITC calorimetry. The binding energy indicated at ~535-fold increase in the association constant (Ka) of the designed protein compared to the EhCaBP1-Wt. Furthermore, we used X-ray crystallography to understand the changes at the atomistic level leading to changes in the functional behavior of EF-hand motif in terms of calcium binding. Surprisingly, we found out the high-resolution structure that diffracted at 1.9Å, showed shrinkage in Ca^{2+} binding coordination sphere resulting in strong coordination yielding high affinity for calcium and forming a hexamer due to the structural changes caused by the designed high affinity calcium sequence.

We present a set of programs (offline) with new scoring functions and a user-friendly webserver (online) to predict, design and engineer EF-hand binding loop. The webserver and the downloadable set of scripts are available at http://202.41.10.46/calb/.

Multicolor Bioluminescent Calcium Imaging Across Three Orders of [Ca^{2+}] Magnitude in Single Living Cells

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Intracellular Ca^{2+} concentration is liked to elaborate regulation of numerous physiological processes from fertilization to apoptosis. Different parts of the cell store, and use, Ca^{2+} at different concentrations, so distinct indicators with different affinities are needed for each compartment. For example, Ca^{2+} is stored in the sarco/endoplasmic reticulum in millimolar amounts and is important for controlling the cytosolic Ca^{2+} level. To study SR/ER Ca^{2+} dynamics, several fluorescent protein based genetically encoded calcium indicators (GECIs) with low Ca^{2+} affinity have been reported. Recently our group reported bioluminescent protein based GECI's with high Ca^{2+} affinity named the green enhanced Nano-lantern Ca^{2+} (GeNL(Ca^{2+})) series (Ca^{2+} Kd 60-520nM). They do not require excitation light and overcome the constraints of fluorescence imaging such as phototoxicity, photobleaching and autofluorescence from the specimen. To investigate Ca^{2+} in the SR/ER by bioluminescence, we have successfully developed a cyan color variant with low affinity named CeNL(Ca^{2+}). In addition, we have developed an orange color variant with intermediate Ca^{2+} affinity, OeNL(Ca^{2+}) that is adapted to mitochondrial Ca^{2+} imaging. The tools can be combined for simultaneous subcellular Ca^{2+} studies across three orders of magnitude [Ca^{2+}] in single cells by expression of ER localized CeNL(Ca^{2+}), mitochondria localized OeNL(Ca^{2+}) and nuclear localized GeNL(Ca^{2+}). SR Ca^{2+} dynamics in C2C12 myoblasts cells has also successfully been studied with the low affinity CeNL(Ca^{2+}) probe.

The Plasma Membrane Calcium Pumps

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The Ca^{2+} ATPases of the plasma membrane (PMCA pumps) play a fundamental role in controlling the homeostasis of Ca^{2+} in all eukaryotic cells. In mammals they are encoded by four separate genes: PMCA1 is ubiquitous and has a housekeeping role; PMCA4 is also ubiquitous but also exerts tissue-specific roles; PMCA2 and PMCA3 are tissue-restricted, with high levels of expression in neurons. The number of PMCA isoforms is greatly increased by alternative splicing of the primary transcripts. The three-dimensional structure of the PMCA pump has not been solved, but molecular modelling on SERCA pump templates reveals that the PMCA pump is organized in the plasma membrane with ten transmembrane helices, two main cytosolic loops and a long C-terminal cytosolic tail. The second loop contains the catalytic center, while the C-terminal domain contains the regulatory calmodulin binding domain, which in the low Ca^{2+} condition of resting state is bound to the main body of the pump, keeping it autoinhibited. When calmodulin is present, it interacts with high affinity with the C-terminal tail and removes it from its binding site(s) in the main body of the pump, restoring activity. As the pump begins pumping Ca^{2+} it lowers its concentration around the cytosolic portion of the pump, promoting the detachment of calmodulin: The activation of the pump by calmodulin is thus by necessity oscillatory. PMCA is also regulated by acidic phospholipids, kinases, dimerization and a series of protein partners. It coexists with much more powerful systems that clear Ca^{2+} from the bulk cytosol (the SERCA pump and the sodium/calcium exchanger of the plasma membrane), and thus has only minor importance in the bulk regulation of cytosolic Ca^{2+}. Its essential role is the regulation of Ca^{2+} homeostasis, and thus of Ca^{2+} signals, in selected sub-plasma membrane microdomains where other important Ca^{2+} related enzymes also reside. PMCA dysfunctions, frequently linked to genetic mutations, are responsible for a number of pathologies, those related to the neurons-restricted isoforms being the best characterized.

A Pathogenic V1143F Mutation in the Neuronal-Restricted Isoform 2 of the PMCA Pump is Linked with Ataxia in Humans

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The fine regulation of intracellular calcium is fundamental for all eukaryotic cells. In neurons, in particular, Ca^{2+} oscillations are involved in the synaptic development, the release of neurotransmitters and the expression of several genes. Not surprisingly, alterations of Ca^{2+} homeostasis were also found to play a pivotal role in the neurodegenerative progression. The peculiar importance of Ca^{2+} signalling in neurons demands the activity of Ca^{2+} pumps (and exchangers) in the restoration of physiological cytosolic concentration of the cation after its increase induced by stimulation. The plasma membrane Ca^{2+} ATPases (PMCA pumps) contribute to the global regulation of Ca^{2+} in the neuronal cytosol, but are particularly important in the regulation of Ca^{2+} homeostasis in selected sub-plasma membrane microdomains where a number of Ca^{2+}-related enzymes that are important for cell function also reside. In mammals the PMCA pumps are encoded by four separate genes and are characterized by different tissue distribution: PMCA 1 and 4 are ubiquitously expressed while PMCA types 2 and 3 are restricted to a limited number of tissues, the most important being the nervous system. Interestingly, diverse mutations of the isoform 3 of the PMCA pump have been associated to X-linked congenital cerebellar ataxias, while malfunctions of the isoform 2 have instead been linked to hearing loss in a digenic process in which mutations in the Ca^{2+} binding cadherin 23 also occur. Here we report a missense mutation (V1143F) in the C-terminal calmodulin binding domain of isoform 2 of the pump in a patient with congenital cerebellar ataxia but with no hearing loss. Biochemical studies have revealed that the mutation impairs the Ca^{2+} ejection function of the pump after stimulation with an inositol-trisphosphate (InsP(3))-linked plasma membrane agonist, leading to cytosolic calcium overload.

A Novel PMCA3 Mutation in an Ataxic Patient with Hypomorphic Phosphomannomutase 2 (PMM2) Heterozygote Mutations: Biochemical Characterization of the Pump Defect

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The neuron-restricted isoform 3 of the plasma membrane Ca^{2+} ATPase plays a major role in the regulation of Ca^{2+} homeostasis in the brain, where the precise control of Ca^{2+} signaling is essential. Several function-affecting genetic mutations in the PMCA3 pump have indeed been described in X-linked congenital cerebellar ataxias. Interestingly, the frequently co-occurring mutations in additional genes suggest a synergistic action in generating the neurological phenotype. Here we report a novel PMCA3 mutation (G733R) in the catalytic P-domain of the pump in a patient affected by non-progressive ataxia, muscular hypotonia, dysmetria and nystagmus. Biochemical studies of the pump overexpressed in model cells have revealed impaired ability to control cellular Ca^{2+} handling both under basal and under stimulated conditions. A combined analysis by homology modeling and molecular dynamics has revealed a role for the mutated residue in maintaining the correct 3D configuration of the local structure of the pump. Mutation analysis in the patient has revealed two additional function-impairing compound heterozygous missense mutations (R123Q and G214S) in the phosphomannomutase 2 (PMM2), a protein that catalyzes the isomerization of mannose 6-phosphate to mannose 1-phosphate. These mutations had been associated to Type Ia congenital disorder of glycosylation (PMM2-CDG), the most common group of disorders of glycosylation of N-linked oligosaccharides. The findings confirm the association of PMCA3 mutations to cerebellar ataxia and strengthen the possibility that PMCAs act as digenic modulators in neuronalCa^{2+}-linked pathologies.

Poster 42: see Session 1–5 on Page 7

Poster 43: see Session 2–4 on Page 11

Poster 44: see Session 2–5 on Page 12

Poster 45: see Session 3–4 on Page 16

Poster 46: see Session 3–5 on Page 17

Poster 47: see Session 4–3 on Page 20

Poster 48: see Session 4–4 on Page 21

Poster 49: see Session 5–4 on Page 25

Poster 50: see Session 5–5 on Page 26

Poster 51: see Session 6–4 on Page 30

Poster 52: see Session 9–4 on Page 41

Poster 53: see Session 9–5 on Page 42

Poster 54: see Session 10–4 on Page 46

Poster 55: see Session 10–5 on Page 47